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# ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA





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# INDEX

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# INDEX

## VOL. 56 FASC. 1-4 1962

## SUPPLEMENTA

*FROM LABORATORIUM FOR PATOLOGI, AUTOPSIAVDELINGEN, RIKSHOSPITALET, OSLO (HEAD PROF OLAV TORGERSEN)*

# INTRASPINAL HEMORRHAGES IN NEWBORNS

*By*

KJELL M ELGJO

Received 27 i 62

Although the importance of intracranial haemorrhages as a cause of neonatal deaths has been generally accepted, little attention has been given to intraspinal haemorrhages The conclusion to be drawn from the few reports published is that intraspinal haemorrhages occur with greater frequency than generally realized (5, 6, 7, 8) The most extensive study in this field has been made by *Hausbrandt & Meier* (5) who found intraspinal haemorrhages in 31 cases in a series of 103 autopsies Most of these were located in the cervical part of the spine In our department we have occasionally observed intraspinal haemorrhages in newborns, and as little attention has been given to this side of paediatric pathology we have found a renewed investigation in this field worth while

## ANATOMY

The vertebral vein system is a plexiform network which cranially communicates with the cranial sinuses and caudally with the venous plexus around the caudal vertebrae The vertebral veins are usually divided into 3 *groups The first* group consists of internal veins surrounding the dura mater within the spinal canal The second group comprises the veins within the bones of the vertebrae, and the third is represented by a venous plexus external [illegible]

The internal group contains [illegible] by far larger than required for [illegible] three systems are interconnecte[illegible] generally arranged longitudina[illegible] versal course The dense network of transversal vessels is interrupted only corresponding to the intervertebral disks The vertebral venous plexus is connected with both the inferior and the superior vena cava and as it is a valveless system with a large capacity it forms an important shunt [illegible]

th [illegible]
In [illegible]
In [illegible]
fr [illegible]
ce [illegible]

## METHOD AND MATERIAL

The present investigation includes 33 successive autopsies of children who died within the 4 first days of life. Two of the infants were stillborn, 25 were premature and 8 were fullborn. Apart from the two who were stillborn all had presented signs of neonatal asphyxia with periods of apnoea and cyanosis.

A routine autopsy was first performed. Subsequently the spinal column was removed in toto and fixed in 4 per cent formalin. After decalcification the column was opened from the dorsal side in such a way that the spinal medulla was disclosed. The ventral ⅔ of the column with the spinal cord was embedded in paraffin and sectioned in the frontal plane. The slides were stained with haematoxylin and eosin. The most important findings by routine autopsy are presented in Table 1.

TABLE 1

*Findings at Routine Autopsy*

| | |
|---|---|
| Pulmonary atelectasis | 19 cases |
| Hyaline membrane disease | 10 |
| Congenital heart anomalies | 2 ,, |
| Erythroblastosis | 2 |
| Bronchopneumonia | 1 ,, |
| Haemorrhages outside the central nervous system | 6 |

## RESULTS

By microscopic examination extradural haemorrhages were found in 23 cases. The size of the haemorrhages varied from small perivascular extravasations to large effusions. In 14 cases small extravasations were found scattered through the greater part of the extradural space. In the remaining 9 cases the haemorrhages were located to the cervical (or upper thoracic) and/or the lower lumbar part of the column. In three cases the haemorrhages extended through the intervertebral foramina and surrounded the nerve roots.

Subarachnoidal haemorrhages were seen in 5 cases. In one additional case several small perivascular haemorrhages were observed in the cervical part of the spinal cord.

An incidental finding was made in one case, a premature child 44 cm long, presenting a congenital anomaly with block formation of 5 vertebrae in the thoracic region.

## DISCUSSION

The postmortem findings were correlated with the information obtained about the mode of delivery and the postnatal state. It appeared that delivery traumata were reported in all of the cases involving subarachnoidal bleeding. The stillborn infants belonged to this category and signs of life had disappeared during delivery in both of the cases. In one of these infants the routine autopsy failed to reveal any pathological findings which could explain the sudden death. In the other one adrenal as well as intracranial haemorrhages were present. Of the remaining 3 cases with intraspinal subarachnoidal haemorrhage forceps

*Fig 1*

Perivascular and diffuse haemorrhages in the extradural space in a premature infant 44 cm long who also had intraventricular and subarachnoidal haemorrhages





*Fig 2*

Multiple small perivascular extravasations in the cervical medulla in a fullborn infant delivered by forceps because of signs of intrauterine asphyxiation

had been used in one case because of protracted birth, one was born in foot ling presentation and the third was born prematurely due to ablatio placentae

All of the infants presented signs of neonatal asphyxia and the great frequency of extradural haemorrhages may be related to capillary damage caused by asphyxiation

The intracranial haemorrhages have been separated into 2 types (2, 3, 4), the first comprising diffuse meningeal haemorrhages (laceration of tentorium cerebelli, laceration of falx cerebri, convexity haemorrhages), the second one intracerebral and circumscribed leptomeningeal haemorrhages The first type seems to occur in full term infants following foot ling or breech delivery, use of forceps and in cases where the mothers are more than 30 years old The second type is seen mainly in premature infants and in infants with neonatal asphyxia Thus the intracerebral and the small leptomeningeal haemorrhages have been related to asphyxia which combined with incompletely developed and feeble vessel walls apparently increases the danger of perivascular haemorrhages

It is probable that the same factors also play an important rôle in the genesis of the intraspinal haemorrhages, and fluctuations in the venous pressure during delivery may be considered as an additional factor Probably also the purely mechanical factors during birth may influence the distribution of the extradural haemorrhages as these most frequently were found in the areas of the spinal column mostly subjected to distorting movements during delivery, *i e* the lower lumbar and the upper thoracic and cervical segments The great frequency of haemorrhages in the presented series may therefore well be caused by various factors acting together, such as prematurity, asphyxiation and mechanical traumata

However, the present series does not allow for any conclusions to be drawn as regards the frequency with which intraspinal haemorrhages occur during and after birth in general, but this investigation suggests that such haemorrhages are no rare occurrences The smaller, extra dural haemorrhages would probably soon be resorbed without giving any clinical symptoms On the other hand it is possible that the haemorrhages per se may deteriorate the condition of an infant already handicapped by prematurity and respiratory difficulties Where the haemorrhages surround the nerve roots passing through the intervertebral foramina it is also possible that this may be followed by an impaired function of the nerve Our series may be regarded as selected due to the relatively great number of premature infants who come to autopsy in our department This fact as well as the small number of cases reduces the validity of any conclusions drawn Further investigations in this field seem, however, to be indicated

## SUMMARY

In a series of 33 successive autopsies of children who died within the 4 first days of life intraspinal haemorrhages were found in 23 cases. *The relation between haemorrhages, mode of delivery and neonatal* condition is discussed.

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6 [illegible]
7 [illegible]
8 [illegible]

*FROM THE DEPARTMENT OF PATHOLOGY, THE FINSEN INSTITUTE AND RADIUM CENTRE COPENHAGEN (DIRECTOR J CLEMMESEN, M D)*

# A CASE OF CARCINOMA ADENOIDES CYSTICUM ARISING IN THE SPHENOID SINUS

*By*

PETER MEYER

Received 6 ii 62

Among tumours originating in salivary-gland tissue there are a few whose peculiar histology and characteristic clinical course have given rise to numerous aetiological hypotheses, and yet their genesis still remains obscure This applies to mixed tumours as well as to adenolymphoma and carcinoma adenoides cysticum These tumours occur chiefly in the large salivary glands, but may also originate in other glands of the upper digestive tract and air passages Frequently they are located in the lateral wall of the nasal cavity, including the maxillary sinus, and in the rhinopharynx, far less commonly in the frontal or sphenoid sinuses which normally hold only a few glands of little activity

Carcinoma adenoides cysticum (c a c ) is a rare tumour Its histological architecture is peculiar, made up of islets of small, densely placed, hyperchromatic cells of the basal type surrounding a cystic cavity with eosinophilic secretion The islets are situated in a more or less hyalinized connective tissue The names applied to this form of tumour in the course of time reflect the hypotheses on its genesis *Billroth* (1859) used the term cylindroma, *Krompecher* (1918) basalioma and others, int al *Barnwater* (1931) endothelioma It was not until 1935 that *Ahlbom* isolated c a c as a special type of carcinoma, believing that it was derived from the excretory ducts

*Ringertz* (1938) in a series of tumours arising in the nose and nasal sinuses, found a total of 8 cases of c a c in addition to 4 benign cystic basal cell tumours From the literature he collected also 52 cases of c a c arising in the nose, maxillary sinuses, and ethmoid cells According to modern criteria, he found several of these reported cases to be doubtful, as c a c was not isolated as a special type until 1935 – C a c arising in the frontal or sphenoid sinuses has not been reported Since, however, tumours arising in the upper posterior part of the maxillary sinuses, the posterior ethmoid cells, or in the sphenoid sinus are indistinguishable from each other in the advanced stage because of early bony destruction, some tumour in the maxillo ethmoid

region may have originated from the sphenoid sinus *Geschickter* (1935) described 15 cases of c a c one of which extended intracranially from the rhinopharynx *Godtfredsen* (1944) reported 5 cylindromas arising in the rhinopharynx, 4 of which showed intracranial invasion *Vald Poulsen* (1953) described 2 adenomatous carcinomas in the ethmoid sinus, both invading the dura and one of them eroding the left cavernous sinus

The symptoms and signs of malignant tumours arising supero-posteriorly in the lateral wall of the nose are fairly characteristic, varying but little with the type of tumour Swelling of the nose or rhinopharynx is an early sign Intracranial extension is rare, but if present it is often perineural through the foramen ovale, foramen rotundum, or foramen lacerum The symptoms from the cranial nerves often occur in a particular sequence The initial symptom will be neuralgia affecting the 2nd trigeminal branch which makes the patient consult the dentist (*Öhngren* 1933) At an early stage all the trigeminal branches and the nerves of the ocular muscles become involved *Godtfredsen* described as a special, rather uncommon syndrome, simultaneous involvement of the trigeminal nerve, nerves of the ocular muscles, and hypoglossal nerve C a c is reported to show a fairly marked tendency to infiltrative growth, often multifocal, but with late metastasization Its growth is mainly extradural

The present case of c a c is of interest for several reasons: Most probably it arose in the sphenoid sinus, it showed pronounced invasive growth with monstrous changes in the base of the skull and invasion of the central nervous system The history was short and atypical, giving rise to a number of diagnostic difficulties

## CASE REPORT

Radium Centre 108016 Path Dept 95/61

A housewife aged 63 In 1952 syphilis was diagnosed and treated in May 1960 cholecystectomy Otherwise in good health in particular she was not very prone to colds or sinusitis

In June 1959 mild paraesthesiae in the left half of the tongue One month later paraesthesiae reduced sensibility and sense of taste in the right half of th[illegible]
In August 1960 [illegible]

[illegible] tongue The patient was admitted [illegible]
hospital where right [illegible]
right accessory n[illegible]
skull were norma[illegible]
ventricle The pat[illegible]
was submitted, [illegible]
posterior cranial [illegible]
The dura coverin[illegible]
No abnormality w[illegible]
One m[illegible]
le[illegible]
no [illegible]
Subsequent

months total paresis of all of the muscles of the right eye developed The patient was now suffering from right sided headache difficulty of walking and vertigo of a gyratory type

In July 1961 she was re admitted now with hard non tender lymph nodes below the right mandible and in the right supra clavicular fossa X rays showed advanced destruction of the base of the skull from the orbit to the foramen magnum A biopsy from the swelling in the right temporal region revealed carcinoma adenoides cysticum and the patient was transferred to the Radium Centre for X ray therapy

With increasing signs of cerebral involvement she died on Aug 29th 1961

Thus, the disease had run its course in a little over a year, manifesting itself as successive signs of neurological loss, first involving the 2nd and 3rd trigeminal branches, the hypoglossal and accessory nerves, then the facial nerve, nerves of the ocular muscles, and the first trigeminal branch Signs of elevated intracranial pressure and regional metastases did not occur until at a late stage

*Post-mortem findings* In the right medial and posterior cranial fossae a *coarsely lobulated tumour, 10 × 8 × 6 cm, inhomogeneous on section,* in places necrotic and invading blood vessels Pronounced destruction



*Fig 1*

... brain stem removed dura intact except ... the right from the roof ... right squama temporalis

*Fig 2*

Photomicrograph of the relation of the tumour to the rhinopharynx Hyalinized connective tissue between the mucous membrane and the polymorphous tumour masses



*Fig 3*

Invasion of the molecular layer of the cerebellar cortex
Nerve cells partially necrotic

*Fig 4*

Islet of typical tumour cells In places imitation of glandular lumina

*Fig 5*

Metastases to the fatty tissue surrounding the right carotid sheath

of bone The tumour was chiefly extradural, but in a few places it had invaded the dura and the central nervous system

Its demarcation was as follows: Anteriorly it had invaded the orbit, the posterior ethmoid cells, and the maxillary sinus which was not, however, quite filled Medially, the tumour exceeded the midline, destroying the entire body of the sphenoid bone and the right half of the basilar part of the occipital bone The cavernous sinus was destroyed, the pituitary gland intact, entirely surrounded by tumour tissue Laterally the tumour extended to the lateral wall of the medial and posterior fossae, destroying the bone and invading the temporal muscle The medial part of the pyramid was destroyed, while its lateral part with the labyrinth was intact The petrous and the cavernous portions of the internal carotid artery were entirely surrounded by tumour tissue Caudally the tumour extended to the parapharyngeal space, destroying the pterygoid process On the other hand, there was no tumour tissue in the rhinopharynx whose mucous membrane was intact, also on microscopic examination Cranially the tumour extended approx 4 cm past the level of the base of the skull, filling the entire medial fossa, the greater part of the posterior fossa and ensheathing all of the cranial nerves except the first It had made a distinct impression into the brain which was shifted far to the left Posteriorly, at the site of the inferior surface of the cerebellum, small ulcerations in the dura with tumour invasion of the pia

Hard, adherent lymph nodes under the right mandible, in the supraclavicular fossa, and along the vessels of the neck

In the lungs basal congestion No metastases to the thoracic or abdominal organs or lymph nodes

Sequelae of cholecystectomy

*Microscopic examination* The tumour (fig 4) consisted of islets and strands of small, oval to round, epithelial cells with sparse cytoplasm, dark, fairly uniform nuclei, and a number of mitotic figures While peripherally there was little cohesion between the cells, they were more densely placed towards the centre Centrally in the islets in several places irregularly demarcated cavities containing eosinophilic, finely granular secretion or blood The islets were seperated by vascular, connective tissue, infiltrated by lymphocytes – The appearances were highly varied, showing invasion of vessels, bone, muscles, cerebellar tissue (fig 3), choroid plexus, the membranes around the pituitary gland, and the central nervous system The pituitary gland and rhinopharyngeal mucosa (fig 2) were intact

The metastases (fig 5) showed a more monotonous and typical appearance, lying in the specimens available in fatty tissue and in a perineural situation, not in lymph nodes

Summing up, the tumour was the shape of a cone with its top in the sphenoid sinus and its base at the site of the temporal fossa It was

*Fig 4*

Islet of typical tumoı r cells In places ımitatıon of glan lular l mına

*Fıg 5*

Metı tases tı the fatty tıssue surrı ındıng tl ı rıght car tı l heatl

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Islet of typical tumour cells In places imitation of glandular lumina

*Fig 5*

Metastases to the fatty tissue surrounding the right carotid sheath

FROM THE DEPARTMENT OF PATHOLOGY (HEAD J CLEMMESEN M D)
THE FINSEN INSTITUTE AND RADIUM CENTRE COPENHAGEN

# BIOLOGICAL DATA OF THE H A 1 TUMOUR

*By*

BENT L SØRENSEN

Received 19 iii 62

The use of cortisone for breaking down immunity to heterologous tissue is well known also when the heterologous tissue is malignant *Iversen* (1956) injected human tumour cells from ascitic fluid intraperitoneally into cortisone treated mice and observed growth of an ascites tumour fatal to the mice and transplantable to other cortisone treated mice After the 3rd transfer generation however the tumour took in mice which had not been pre treated with cortisone During the past 5 years this ascites tumour which is called H A 1 has been transplanted further to untreated mice by intraperitoneal inoculation of ascites from tumour bearing mice every 10th day

It was found that the tumour was made up of triploid cells having murine chromosomal characteristics from the 15th transfer generation despite the human origin of the tumour cells (*Iversen* 1958 a)

In X ray irradiated rats the H A 1 tumour also grows (*Iversen* 1958 b) and tolerance to the tumour cells is obtained in rats treated during foetal life with H A 1 tumour cells (*Iversen & Sorensen* 1960)

An investigation of the findings relating to the growth of the H A 1 tumour has not been submitted previously The survival time of the transplanted animals will be analysed from the point of view of possible alterations in the malignancy of the tumour Other factors included in the analysis are the growth rate and the duration of the individual generation of the tumour cells as well as the duration of mitosis

## MATERIAL AND METHODS

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## RESULTS

### A Survival Time of Mice with the H A 1 Tumour

During two consecutive periods of 6 months each the survival time of the routinely transplanted male mice was analysed and the variance

highly destructive, invading vessels, but had little tendency to metastasize Owing to the widespread bone destruction, it is impossible to locate the origin exactly, but the sequence of the symptoms and signs, the intact rhinopharyngeal mucosa, and the absence of tumour tissue in the *anterior part of the maxillary sinus indicate an origin from the sphenoid sinus* — It has presumably first spread extradurally to the lateral and posterior aspect along the inferior petrosal sinus to the jugular foramen Later, it has propagated on both sides of the right pyramid which was not destroyed until a late stage The marked spread in the posterior fossa and the invasion of the dura at this site were possibly due to the right-sided posterior craniotomy which has partially broken down the dural barrier at the site of the foramen magnum

## SUMMARY

This is a description of a short, atypical history of carcinoma adenoides cysticum, which probably arose in the sphenoid sinus, destroying the base of the skull

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The variances on the mean survival times of the mice in the two 6-month periods do not differ significantly, whereas the mean survival times following transplantation are significantly different the survival time following transplantation becoming shorter the larger the number of transfer generations the tumour has passed through

The number of tumour cells injected was not known at the routine transplantation But it has been stated by *Goldberg Klein & Klein* (1950) that the survival time with Ehrlich s ascites tumour is almost independent of the inoculated number of cells, once a certain minimum number has been exceeded To assess this possible minimum value of H A 1 tumour cells different numbers of tumour cells were injected intraperitoneally into Bagg mice and their survival times analysed as shown in Table 2 and Fig 2

TABLE 2

*Survival Time of Bagg Mice Transplanted with Varying Numbers of H A 1 Tumour Cells*

| Injected number of cells × $10^6$ | 1 | 2 | 5 | 10 | 20 | 30 |
|---|---|---|---|---|---|---|
| Number of takes/number of mice | 4/5 | 4/5 | 5/5 | 5/5 | 5/5 | 5/5 |
| Mean survival in days | 23 9 | 25 0 | 22 4 | 18 6 | 16 2 | 14 0 |
| Standard deviation | 10 3 | 6 5 | 6 1 | 3 2 | 2 2 | 1 4 |

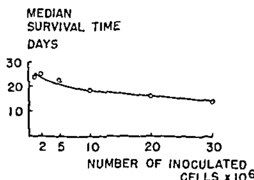



Fig 2

Relation between survival time and the number of inoculated tumour cells

From Fig 2 it will be seen that between 10 × $10^6$ and 30 × $10^6$ the curve is least steep while less than 10 × $10^6$ tumour cells increase the survival time somewhat more In the routine transplantation approx 25 × $10^6$ tumour cells were injected (S D = 5 × $10^6$), *i e* cell numbers so great that a considerable divergence from this number does not, according to Fig 2 cause any major difference in the survival time of the transplanted mice

B *Weight of Tumour Bearing Mice*

Fig 3 shows the percentage weight gain of transplanted male and female mice

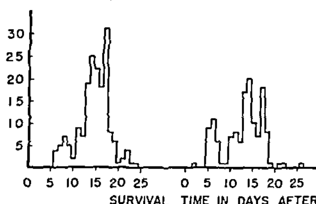



*Fig 1*

*Variation in survival time of transplanted mice The histogram to the left represents the 125th to the 143rd transfer generation that to the right the 144th to the 162nd transfer generation*

of the mean survival time calculated (Table 1) Thereupon a statistical comparison was carried out using the t test Such a calculation is justified *as the survival time of the mice is distributed with approximation* according to a normal Gaussian curve as shown in Fig 1

TABLE 1

*Mean Values and Variance of Survival Time of Tumour Bearing Mice*

| | | |
|---|---|---|
| Transfer generation | 125 to 143 | 144 to 162 |
| No of mice n | 178 | 134 |
| Mean survival $X_m$ | 14.47 days | 12.71 days |
| Sum of survival time $\Sigma X$ | 2575 days | 1703 days |
| $(\Sigma X)^2$ | 6630625 | 2900209 |
| $\Sigma X^2$ | 39863 | 24187 |
| Variance s | 14.67 = 3.8 | 18.99 = 4.42 |

The mean survival time in the two periods was compared statistically by the t test First the standard deviations were compared Mean *error on the standard deviations* $\left(e = \frac{s}{\sqrt{2n}}\right)$

$$e_1 = 0.04 \qquad e_2 = 0.102$$

Mean error on the difference of the standard deviations

$$e_d = \sqrt{e_1^2 + e_2^2} \quad \sqrt{0.1424} \quad 0.4$$

$$t = \frac{s - s_1}{e_d} = \frac{0.6}{0.4} = 1.5 < 2.58 \text{ (not significant)}$$

Then the mean values in the two periods were compared

$$t = \frac{X_{m1} - X_m}{e_d} = \frac{1.76}{0.48} = 3.7 > 2.58 \text{ (significant)}$$

The variances on the mean survival times of the mice in the two 6 month periods do not differ significantly, whereas the mean survival times following transplantation are significantly different, the survival time following transplantation becoming shorter the larger the number of transfer generations the tumour has passed through

The number of tumour cells injected was not known at the routine transplantation But it has been stated by *Goldberg, Klein & Klein* (1950) that the survival time with Ehrlich's ascites tumour is almost independent of the inoculated number of cells, once a certain minimum number has been exceeded To assess this possible minimum value of H A 1 tumour cells different numbers of tumour cells were injected intraperitoneally into Bagg mice and their survival times analysed as shown in Table 2 and Fig 2

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| Injected number of cells × 10⁶ | 1 | 2 | 5 | 10 | 20 | 30 |
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| Mean survival in days | 23 9 | 25 0 | 22 4 | 18 6 | 16 2 | 14 0 |
| Standard deviation | 10 3 | 6 5 | 6 1 | 3 2 | 2.2 | 1 4 |

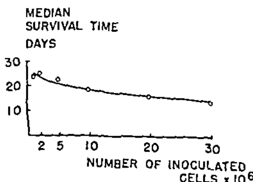



*Fig 2*

Relation between survival time and the number of inoculated tumour cells

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B *Weight of Tumour Bearing Mice*

Fig 3 shows the percentage weight gain of transplanted male and female mice

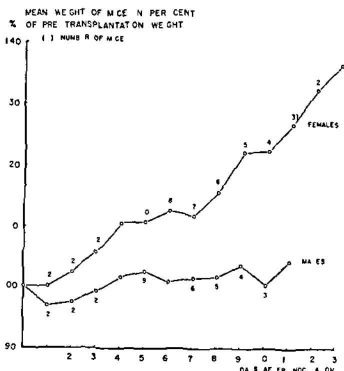



*Fig 3*
*Mean weight of mice in per cent of weight at the time of transplantation*
The figures in brackets indicate the number of mice in each analysis

*It is notable that the weight of the females increased considerably* more than that of the males due to the fact that the quantity of ascites increased more in the females (cf Fig 4)

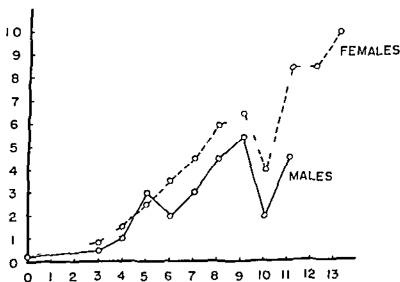



*Fig 4*
*Quantity of ascites in relation to time after transplantation*

The body weight after removal of the ascites tumour by aspiration is given in Fig 5 which shows that the mice lose much weight This explains why the total weight of the males may be almost unchanged (Fig 3) despite growth of the tumour

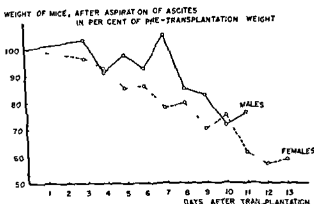



*Fig 5*

Body weight of tumour bearing mice after aspiration of ascitic fluid in per cent of pre transplantation weight

## C *Investigation of the Growth Curve of the H A 1 Tumour*

It is difficult to follow the number of tumour cells in the same mouse day by day, as the repeated tappings may be imagined to influence the quantity of ascites as pointed out by *Revesz & Klein* (1954) Adding a new mouse every day introduces the dispersion which is caused by biological variations Moreover, it is not the tumour growth curve for the individual mouse which is of interest, but growth curves representative of male and female mice separately for purposes of comparison

From the 3rd day after transplantation one male and one female mouse was killed by decapitation every day, and the number of tumour cells found by multiplying the concentration of cells with the quantity of ascites Males were followed in this way up to the 11th day and females up to the 13th day after transplantation (Table 3)

The concentration of cells does not appear to be related to the time elapsing after transplantation In Table 4 the coefficient of variation on the concentration of tumour cells is calculated and found to be 20 per cent of the mean value

On the basis of the values representing number of tumour cells in relation to time after transplantation the tumour growth curve is plotted on Figs 6 and 7, the logarithmic number of cells being the ordinate and the time after transplantation the abscissa

In the case of the males the total number of tumour cells from the 3rd to the 9th day is distributed around a straight line in Fig 6 and in the case of the females in Fig 7 around the straight line from the

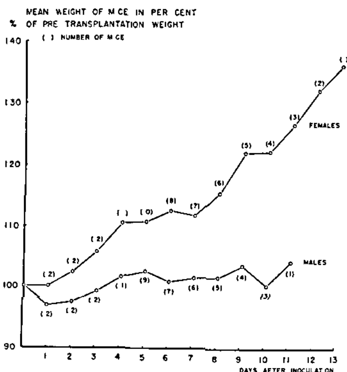



*Fig 3*

Mean weight of mice in per cent of weight at the time of transplantation The figures in brackets indicate the number of mice in each analysis

It is notable that the weight of the females increased considerably more than that of the males due to the fact that the quantity of ascites increased more in the females (cf Fig 4)

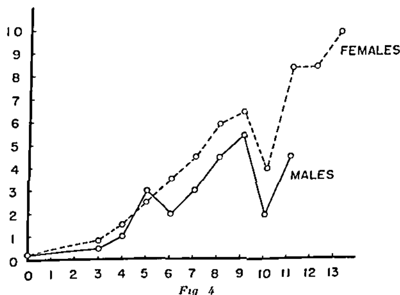



*Fig 4*

Quantity of ascites in relation to time after transplantation

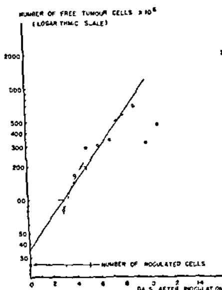



*Fig 6*

Relation between log number of tumour cells in the peritoneal cavity and time after transplantation Male mice

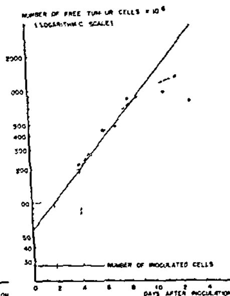



*Fig 7*

Relation between log number of tumour cells in the peritoneal cavity and the time after transplantation Female mice

logical variation definitely predominates over the counting error In calculating the regression coefficients of the curves, therefore, it is enough to consider the mean values plotted on the curves

The equation for the solid line in Fig 6 is then $y = 0\,141\,x + 7\,598$ ($y$ = number of days after transplantation $x$ = logarithmic number of cells)

The equation for the solid line in Fig 7 will be $y = 0\,126\,x + 7\,804$

The doubling time (the time it takes a cells to become 2 a cells) = the generation time G may be calculated from the regression coefficients

$$G_{male} = \frac{0\,3010}{0\,141} \times 24 \text{ hours} = 51\,23 \text{ hours} \quad (0\,3010 = \log 2)$$

$$G_{female} = \frac{0\,3010}{0\,126} \times 24 \text{ hours} = 57\,33 \text{ hours}$$

These generation times may be read from the curves in Fig 6 and Fig 7 by means of the vertical and horizontal dotted lines which in Fig 7 show that $100 \times 10^6$ cells correspond to the time 2 days, and double this number, $200 \times 10^6$ cells correspond to the time approx 4 days The generation time then, is approx (4 days–2 days) = 2 days

By means of the regression coefficients b and their variance V, which is

$V_{(b\,male)} = 0\,000266$ and $V_{(b\,female)} = 0\,000414$

4th to the 9th day after transplantation In other words the growth of the tumour during these periods is exponential

TABLE 3

*Concentration of Tumour Cells and Quantity of Ascites in Bagg Mice in Relation to the Time of Transplantation*

| Sex | Days after transpl | ml ascites | cells per cu mm | Total number of cells |
|---|---|---|---|---|
| Male | 0 | 0 2 | 137 500 | 27 50 × $10^6$ |
| Male | 3 | 0 5 | 159 185 | 79 59 × $10^6$ |
| Male | 4 | 1 0 | 163 835 | 163 84 × $10^6$ |
| Male | 5 | 3 0 | 97 650 | 292 95 × $10^6$ |
| Male | 6 | 2 0 | 153 605 | 307 21 × $10^6$ |
| Male | 7 | 3 0 | 113 615 | 340 85 × $10^6$ |
| Male | 8 | 4 5 | 121 985 | 548 93 × $10^6$ |
| Male | 9 | 5 5 | 125 860 | 692 23 × $10^6$ |
| Male | 10 | 2 0 | 161 820 | 323 64 × $10^6$ |
| Male | 11 | 4 5 | 104 315 | 469 42 × $10^6$ |
| Female | 0 | 0 2 | 137 500 | 27 50 × $10^6$ |
| Female | 4 | 1 5 | 139 755 | 209 64 × $10^6$ |
| Female | 5 | 2 5 | 103 385 | 258 46 × $10^6$ |
| Female | 6 | 3 5 | 124 620 | 436 17 × $10^6$ |
| Female | 7 | 4 5 | 105 090 | 472 91 × $10^6$ |
| Female | 8 | 6 0 | 136 400 | 818 40 × $10^6$ |
| Female | 9 | 6 5 | 128 030 | 832 20 × $10^6$ |
| Female | 10 | 4 0 | 64 635 | 258 54 × $10^6$ |
| Female | 11 | 8 5 | 110 515 | 939 38 × $10^6$ |
| Female | 12 | 8 5 | 152 210 | 1293 79 × $10^6$ |
| Female | 13 | 10 0 | 77 655 | 776 55 × $10^6$ |

The slope of the straight lines was subjected to regression analysis (Figs 6 and 7) as described by *Hald* 1948 This showed that the variance on the cell counts around their mean values was 0 0034 in the males and 0 0023 in the females or of the same order of magnitude

TABLE 4

*Variation in Concentration of Tumour Cells in Ascitic Fluid from the 3rd to the 13th Day after Transplantation*

| | No of mice | Mean M | S D | | Maximum and minimum | |
|---|---|---|---|---|---|---|
| | | | Total | per cent of mean | Actual | Calculated M + 2 85 S D |
| Cells per cu mm | 21 | 1251198 | 25309 | 20 2 | 163835<br>64635 | 197328<br>53068 |

The scatter of the mean values around the two straight lines indicates the biological variation which was found to be 0 075 for the males and 0 033 for the females That is these values too are of the same order of magnitude but one order of magnitude in excess of the variance of the individual counts around their mean value In other words the bio

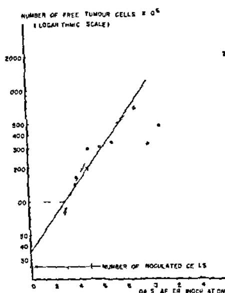



*Fig 6*

Relation between log number of tumour cells in the peritoneal cavity and time after transplantation Male mice

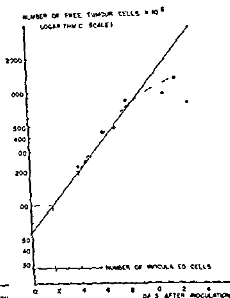



*Fig 7*

Relation between log number of tumour cells in the peritoneal cavity and the time after transplantation Female mice

logical variation definitely predominates over the counting error In calculating the regression coefficients of the curves, therefore, it is enough to consider the mean values plotted on the curves

The equation for the solid line in Fig 6 is then $y = 0\,141\,x + 7\,598$ ($y$ = number of days after transplantation $x$ = logarithmic number of cells)

The equation for the solid line in Fig 7 will be $y = 0\,126\,x + 7\,804$

The doubling time (the time it takes a cells to become 2 a cells) = the generation time G may be calculated from the regression coefficients

$$G_{male} = \frac{0\,3010}{0\,141} \times 24 \text{ hours} = 51\,23 \text{ hours} \quad (0\,3010 = \log 2)$$

$$G_{female} = \frac{0\,3010}{0\,126} \times 24 \text{ hours} = 57\,33 \text{ hours}$$

These generation times may be read from the curves in Fig 6 and Fig 7 by means of the vertical and horizontal dotted lines which in Fig 7 show that $100 \times 10^6$ cells correspond to the time 2 days, and double this number $200 \times 10^6$ cells correspond to the time approx 4 days The generation time then is approx (4 days 2 days) = 2 days

By means of the regression coefficients b and their variance V, which is

$$V_{(b\text{-}male)} = 0\,000266 \text{ and } V_{(b\ female)} = 0\,000414$$

it may be calculated by the t test whether the regression coefficients differ significantly, as

$$t = \frac{b_{male} - b_{female}}{\sqrt{V_{(b\ male)} + V_{(b\ female)}}} = \frac{0.015}{\sqrt{0.000680}} = 0.58$$

which is not significant with 9 degrees of freedom

In other words, the investigations did not demonstrate any sex difference in the growth rate of the H A 1 tumour

Therefore, we can consider the two sets of values in Figs 6 and 7 as belonging to the same group and calculate the common regression coefficient which is 0 135

The standard deviation on the regression coefficient

$$b_{common} = \sqrt{\frac{s^2}{\Sigma (x - \bar{x})^2}} = \sqrt{\frac{0.00699}{46}} = 0.012$$

$$b_{common}\ (5\ \text{per cent uncertainty}) = 0.135 \pm 2.26 \times 0.012 = \begin{cases} 0.162 \\ 0.108 \end{cases}$$

$$\text{The corresponding generation time} = \frac{0.3010}{0.135} \times 24\ \text{hours} =$$

$$53.51\ \text{hours} \begin{cases} \text{max}\ 66.89\ \text{hours} \\ \text{min}\ 44.49\ \text{hours} \end{cases}$$

## D *Calculation of Mitosis Time*

Concurrently with the investigation of the generation time of the tumour cells by counting the cells from the 3rd to the 11th day after transplantation, smears of the ascitic fluid were made from the same mice After nuclear staining with gallocyanin the mitoses were counted The ascitic fluid was always removed at the same hour of the day in order to avoid a possible influence of a variation in the mitotic numbers due to a diurnal cycle as described by *Bullough* (1948) in normal mouse tissue and by *Freymann* (1956) in Yoshida ascites tumour

From each mouse 10,000 tumour cells were counted, and the mitotic index MI represents the number of mitoses found divided by 10,000 At the same time, differential counts of the mitoses were made, the mitotic phases being as suggested by *Maximow & Bloom* (1948)

(1) early prophase, the spireme stage with preserved nuclear membrane
(2) late prophase, the spireme stage without nuclear membrane,
(3) metaphase,
(4) anaphase, the chromosomes being drawn towards the two centrioles,
(5) telophase, chromosomes breaking up, the nuclear membrane re-forming, and two cells forming by constrictions of the cytoplasm

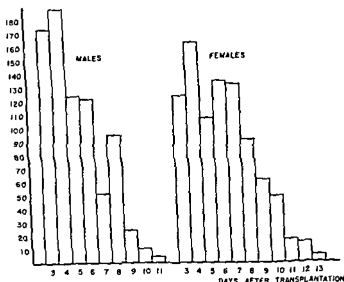



*Fig 8*
Number of mitoses per 10 000 tumour cells in relation to time after transplantation

In fig 8 the number of mitoses is plotted against time after transplantation for males and females separately It was mentioned above that from the 3rd to the 9th day there was an almost logarithmic increase in the number of tumour cells Therefore, one might expect a constant mitotic index during this period But the mitotic index is highest a few days after the transplantation and thereupon decreases The explanation is probably that the increase in the tumour cells is not ideally logarithmic The dotted lines in Figs 6 and 7 also seem to be equally justified and properly determined by the points as the calculated straight lines which indicates that the doubling time of the cells increases with the time after transplantation in consequence of a decreasing mitotic index

Calculation of the mitosis time was carried out as advocated by *Hoffman* (1949) according to which

$$MI = m\,N\,\log_e 2 \times T\,G \qquad (1)$$

($mN$ = *mitotic index, m being the number of mitoses seen among N* cells, T = mitosis time, G = generation time found to be 53 51 hours)

From (1) we derive

$$T = MI \times G \log_e 2 = MI \times 53\,51/0\,693 = MI \times 77\,22$$

Since the increase in the tumour cells is approximately logarithmic, it is natural to use a mean for mitotic indices during this period, i e from the 3rd to the 9th day

As ax stands for the mitotic index on the xth day after transplantation, we get

TABLE 5

*Indices for Mitotic Phases of the Tumour Cells from the 3rd to the 9th Day*

| Male mice | (1/2$a_3$ | + | $a_4$ | + | $a_5$ | + | $a_6$ | + | $a_7$ | + | $a_8$ | + | 1/2$a_9$) : 6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Early prophase | (0.0009 | + | 0.0018 | + | 0.0016 | + | 0.0010 | + | 0.0010 | + | 0.0010 | + | 0.0001) : 6 = 0.0012 |
| Late prophase | (0.0015 | + | 0.0019 | + | 0.0004 | + | 0.0007 | + | 0.0006 | + | 0.0006 | + | 0.0003) : 6 = 0.0010 |
| Metaphase | (0.0029 | + | 0.0068 | + | 0.0043 | + | 0.0080 | + | 0.0026 | + | 0.0052 | + | 0.0005) : 6 = 0.0051 |
| Anaphase | (0.0022 | + | 0.0026 | + | 0.0026 | + | 0.0010 | + | 0.0005 | + | 0.0014 | + | 0.0002) : 6 = 0.0018 |
| Telophase | (0.0014 | + | 0.0056 | + | 0.0034 | + | 0.0014 | + | 0.0004 | + | 0.0012 | + | 0.0002) : 6 = 0.0023 |
| Female mice | | | | | | | | | | | | | |
| Early prophase | (0.0009 | + | 0.0014 | + | 0.0013 | + | 0.0008 | + | 0.0026 | + | 0.0006 | + | 0.0003) : 6 = 0.0013 |
| Late prophase | (0.0007 | + | 0.0024 | + | 0.0013 | + | 0.0013 | + | 0.0018 | + | 0.0008 | + | 0.0003) : 6 = 0.0014 |
| Metaphase | (0.0030 | + | 0.0080 | + | 0.0066 | + | 0.0077 | + | 0.0061 | + | 0.0040 | + | 0.0015) : 6 = 0.0062 |
| Anaphase | (0.0007 | + | 0.0014 | + | 0.0008 | + | 0.0015 | + | 0.0009 | + | 0.0024 | + | 0.0005) : 6 = 0.0014 |
| Telophase | (0.0009 | + | 0.0031 | + | 0.0006 | + | 0.0020 | + | 0.0017 | + | 0.0012 | + | 0.0005) : 6 = 0.0017 |

$$\left(\frac{a_3 + a_4}{2} + \frac{a_4 + a_5}{2} + \frac{a_5 + a_6}{2} + \frac{a_6 + a_7}{2} + \frac{a_7 + a_8}{2} + \frac{a_8 + a_9}{2}\right) : 6$$

which is the mean between the 3rd and the 4th day + the mean between the 4th and 5th day and so on divided by the number of means

In a reduced form

$$(\tfrac{1}{2}\, a_3 + a_4 + a_5 + a_6 + a_7 + a_8 + \tfrac{1}{2}\, a_9) : 6 \qquad (2)$$

Inserting the mitotic indices for male mice in (2)

$$(0.0086 + 0.0187 + 0.0123 + 0.0121 + 0.0051 + 0.0094 + 0.0012) : 6 = 0.0112 \qquad (3)$$

Inserting the mitotic indices for female mice in (2)

$$(0.0062 + 0.0161 + 0.0106 + 0.0133 + 0.0131 + 0.0090 + 0.0009) : 6 = 0.0115 \qquad (4)$$

From the two values (3) and (4) we calculate the mean 0.01135 since as already mentioned there was no significant sex difference in the growth rate

Then the duration of mitosis when MI = 0.01135 is inserted in (1) is

$$T = 0.01135 \times 77.22 \text{ hours} = 0.8764 \text{ hours} = 52.58 \text{ minutes}$$

Paying regard to the previously calculated maximum and minimum values for the generation time we calculate

$T_{max} = 65.73$ min and $T_{min} = 43.87$ min (corresp to the 5 per cent level)

The duration of the individual phases of mitosis is calculated analogously computing the indices of the mitotic phases from the 3rd to the 9th day according to (2) These values are shown in Table 5

Accordingly there is no great difference between the indices in male and female mice Therefore the average values for male and female mice are used for calculating the duration of the various phases of mitosis (Table 6)

TABLE 6

*Average Indices for Male and Female Mice*

| $\frac{\text{Male + female}}{2}$ | Indices |
|---|---|
| Early prophase | 0.00125 |
| | 0.0012 |
| | 0.00565 |
| | 0.0016 |
| | 0.0020 |
| Sum = MI | 0.0117 |

The indices listed in Table 6 are inserted in (1) $MI = \log_e 2 \times T : G$ inserting instead of mitotic index MI prophase index metaphase index etc

Thereupon the following durations of the various phases are found (Table 7)

TABLE 7

*Duration of Mitotic Phases for H A 1 Tumour Cells Absolute Numbers and per Cent*

| | | |
|---|---|---|
| Early prophase | 5 79 min | 10 7 % |
| Late prophase | 5 56 min | 10 2 % |
| Metaphase | 26 18 min | 48 3 % |
| Anaphase | 7 41 min | 13 7 % |
| Telophase | 9 27 min | 17 1 % |
| Sum = duration of mitosis | 54 21 min | 100 0 % |

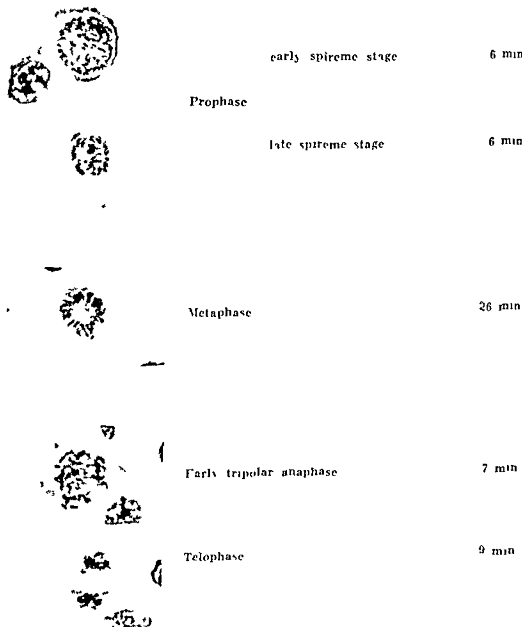



*Fig 9*

H A 1 tumour cells in the phases of mitosis which were differential counted Duration of the individual phases to the right (gallocyanin staining 600×)

The percentages given in Table 7 show in how high a percentage the mitoses are observed in the phases stated and at the same time how high a percentage of the total duration of mitosis the individual phases occupy

Fig 9 illustrates the phases of mitosis and their duration

## DISCUSSION

### A *Survival Time of the Transplanted Mice*

According to *Iversen* (1956) male mice survived for an average of 18 days and female mice for 15 days after the routine transplantation calculated from the 30th to the 40th passage on 50 mice of each sex The present investigation was performed approx 100 passages later or almost 3 years after *Iversen*'s original study In the course of this period the mean survival time of the male mice has decreased to 13 15 days It was not possible to subject this difference to a statistical significance calculation since the variance on the mean survival time is not available from the earliest analyses

The survival time must depend also upon the number of cells injected at the time of transplantation In the present series 0 2 ml ascites was injected but the exact number of cells was not known In the case of the Ehrlich ascites carcinoma *Goldberg Klein & Klein* (1950) have reported that the mean survival time is almost independent of the number of cells inoculated once a certain minimum value—found to be $2 \times 10^6$—of tumour cells is exceeded At an estimate $25 \times 10^6$ tumour cells were used for transplantation of the H A 1 tumour or a number far exceeding the critical number for Ehrlich's ascites carcinoma In the case of the H A 1 tumour Fig 2 showed that less than $10 \times 10$ tumour cells for the transplantation increased the survival time by appreciably more than a corresponding increase in the number of cells shortened the survival time But this value is not nearly as characteristic as the corresponding value for the Ehrlich ascites carcinoma In the routine transplantation of the H A 1 tumour however the number of tumour cells always by far exceeded $10 \times 10^6$ the mean value for the cell concentration being 125 000 cells per cubic mm ascites with a coefficient of variation of 20 *per cent* so that alterations in the quantity of inoculate influence the survival time but slightly It is justified therefore although the number of injected cells is not known to compare the survival times of the Bagg mice after transplantation of H A 1 tumour cells

During the two consecutive 6 month periods there was furthermore a significantly shorter survival *time during the latter period* Thus all the appearances indicate an adaptation of the H A 1 tumour to the present strain of mice with increasing number of transfer generations

### C Growth Curve of the Tumour Cells

The concentration of the tumour cells in the ascitic fluid showed a variation of the same magnitude as in Ehrlich ascites carcinoma according to the investigations of *Goldberg, Klein & Klein* (1950) who found a coefficient of variation of 26 per cent of the mean

A logarithmic increase in the number of cells in expected during the time the tumour grows rapidly, as also found by *Révész & Klein* (1954) Figs 6 and 7, however, show that the injected number of cells is below the calculated straight lines, indicating that during the first days after transplantation there has not been a *period of* slower cell division as the lag phase seen in culturing bacteria on culture media and as reported for other ascites tumours, *e g* by *Klein* (1955), who *found a latent period of two days before the logarithmic cell division* set in

The absence of a latent period immediately after the transplantation might be imagined to be due to the inoculated number of cells, 27 5 × 10⁶, being far in excess of the above-mentioned minimum number, over which an increase does not cause any notable shortening of the mean survival time This is in accord with the fact that *Révész & Klein* (1954) found the latent period to disappear entirely when a large number of tumour cells were injected and to become the more marked the more the number of cells approaches the lowest value which results in takes

The finding of different weight curves in male and female mice (Figs 3 and 4) might give rise to a suspicion of a different mode of tumour growth in male and female mice analogous to the difference in the mean survival time in the two sexes following transplantation of the tumour (*Iversen* 1956)

However, the generation time of the H A 1 tumour cells did not differ significantly for male and female mice So, it may be concluded that the H A 1 tumour grows in the same way in both sexes, but that as hosts male and female mice presumably react to the tumour in different ways

Studying the Ehrlich ascites carcinoma, *Lettré*, in 1943, found the generation time to be approx 24 hours *Klein & Révész* (1953) found the generation time to be approx 24 hours at the beginning of the rapid growth phase and approx 48 hours towards the end of this growth period This alteration in the generation time is contrary to what has been found by *Révész & Klein* (1954) in the three ascites lymphomas which showed exponential growth for some time and a generation time of only 5–10 hours

Accordingly, the generation time of 53 5 hours in the case of the H A 1 tumour is long compared with the findings with other ascites tumours

That this must be a mean generation time may be seen from the falling mitotic index from the 3rd to the 9th day, which is not com-

patible with an ideal exponential increase in the number of tumour cells and a constant generation time during this period Plotting of the curved lines on Figs 6 and 7 showed that the generation time grew longer and longer with the time elapsing after transplantation At an estimate, it may be read from these figures that a doubling of the cell count around the 4th day requires 30 hours and around the 7th day 60 hours This corresponds to the prolongation of the generation time found by *Klein & Révész* (1953) in the case of Ehrlich ascites carcinoma

Therefore, it is compatible with a doubling of the generation time from the 4th to the 7th day that the mitotic index for male and female mice on the 4th day was on an average twice as high as on the 7th day

D *Duration of Mitosis*

[illegible]

and then declines The mitotic index was practically the same in male and female mice, indicating that the tumour cells had the same ability for growth in both sexes The duration of mitosis was of the same order of magnitude as with Ehrlich ascites tumour cells whose mitosis time is 64 minutes (*Klein & Revesz* 1953) The mitosis time for Yoshida sarcoma cells is approx 50 minutes (*Sato, Atsumi, Satoh & Nakamura* 1952) These times are of the same order of magnitude as for normal cells, but far from being among the shortest which are seen in fertilized ova, *e g* in rabbits, in which the mitosis takes only about 10 minutes with an intermitotic period of 8–9 hours (*Hughes* 1952)

In calculating the duration of the individual phases of mitosis there is a factor of uncertainty, *viz* that the earliest stages of prophase may be overlooked in the smear, the observed length of prophase then being interpreted as a minimum value In the case of Yoshida sarcoma cells *Sato, Atsumi, Satoh & Nakamura* (1952) found a similar percental length of prophase as the present author found for the H A 1 tumour cells The metaphase of Yoshida cells was found to make up only 26 per cent of the entire duration of mitosis, as compared with 48 per cent in the H A 1 tumour cells In the latter, telophase occupies 17 1 per cent of the mitosis time, while the telophase of Yoshida cells occupies 42 per cent Compared with other tissues (*Hughes* 1952), the metaphase of H A 1 tumour cells is relatively long There is, however, a source of error, *viz* that the number of cells in telophase on the smears may be found to be too low, as even a cautious smearing of a drop of asc[illegible] often [illegible]

[illegible] phases as too long In other words, the difficulty concerns the counting of initial and terminal stages of mitosis

## SUMMARY

The biological data of the H A 1 tumour are analysed The mean survival time of the tumour-bearing animals was found to be 14 47 days, and 6 months later 12 71 days after the transplantation, so that the tumour cells appear to become more malignant to the host organism the more transfer generations the tumour has passed through Transplanted female mice show a considerable weight gain unlike male mice, and the formation of ascites is also more pronounced in the females The increase in the number of tumour cells appears to be approximately exponential from the 3rd to the 9th day after the transplantation The generation time was calculated to be an average of 53 5 hours during this period Later, it is discussed that the growth of the tumour cells cannot be ideally exponential, and the generation time on the third day is more correctly about 30 hours and on the 9th day about 60 hours In conformity with this increasing generation time the mitotic index of the tumour cells fell with the time elapsing after transplantation The duration of mitosis was calculated to be about 53 minutes

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# INFLUENCE OF THYROXIN ON REGENERATION OF THE LYMPHATIC TISSUE AFTER CORTICOSTEROID INDUCED INVOLUTION

*A Quantitative Study in Guinea-Pigs*

*By*

LARS GYLLENSTEN

Received 19 III 62

It has been known for many years that hyperthyreosis, both experimental and spontaneous, as well as administration of thyroid hormone under suitable conditions, can produce hyperplasia of the thymus and lymphatic tissue (*Hammar* 1936) For a more extensive bibliography, reference is made to earlier studies by the present author and coworkers (*Gyllensten* 1953, *Barnholdt-von Euler et al* 1959, *Ernstrom & Gyllensten* 1959) This effect may possibly be a component of a generalized mitosis-stimulating action of the thyroid hormone (*Leblond & Carriere* 1955)

The ability of the thyroid gland to stimulate growth of lymphatic tissue seems to be part of a homeostatic mechanism which comes into function after operative (*Gyllensten* 1953) or hormonal (*Bodlund & Gyllensten* 1954, *Barnholdt-von Euler et al*) reduction of the thymo-lymphatic system Thus, signs of increased activity were observed in the thyroid gland after such reduction of the thymo lymphatic tissue An additional factor was found to be of importance for regeneration, i e an increased tendency of the involuted tissue to react to raised activity in the thyroid gland by greater growth than that of normal thymo lymphatic tissue Thus, exogenous thyrotropic hormone was observed to produce a greater increase in weight of lymph node tissue in guinea pigs pretreated with cortisone acetate, than in untreated controls (*Barnholdt-von Euler et al*) The participation of thyroid hormone during regeneration of thymo-lymphatic tissue is in conformity with several investigations on regeneration in other organs and tissues of mammals, when thyroid extract has proved to have a stimulating effect A comprehensive list of references has been given by *Needham* (1960)

The expenses of this investigation were partly defrayed by a grant from the *Swedish Cancer Society*

The histological changes in lymphatic tissue after thyroxin treatment have been studied previously by quantitative methods (*Ernstrom & Gyllensten* 1959) *Among the observations were an increase in the number of plasma cells and other pyroninophil cells in the medullary cords of the lymph nodes, as well as in the red pulp of the spleen, during thyroxin-induced growth* In the cortex, there was an increase in number of immature pyroninophil cells (transitional cells) of blast nature Signs of an increased migration of lymphocytes from the lymph node cortex were present, in the form of a relative decrease in the ratio of lymphocytes to reticular cells Studies of the circulating blood lymphocytes *showed no significant change in the number of white cells per volume unit of blood under the influence of thyroxin*, whereas the mitochondria content per lymphocyte, on the contrary, increased greatly during the experimental period, indicating a migration of young lymphocytes (*Ernstrom & Larsson* 1961)

To sum up, it can be said that thyroxin brought about greater maturation in the medullary cords of the lymph nodes, with an increase in the number of plasma cells, as well as of immature cells of blast type *in both cortex and medulla Moreover, the lymph-node tissue* increased in weight, despite a concurrent migration of young lymphocytes into the blood Thus, stimulation of several cytopoietic processes was evident in the lymphatic tissue, *i e*, both maturation and growth processes

In experimental involution after administration of glycocorticosteroid, signs of regeneration also appear in the medullary cords of the lymph *nodes—after an initial stage of a day or so*—with increased maturity of pyroninophil cells, and an increase in transitional cells, i e, cells of blast nature (*Gyllensten* 1962) Consequently, it should be possible, by similar quantitative analyses of the differential histology during regeneration, to obtain some explanation of the way in which thyroxin partakes in this process *Before this can be done, it must be determined whether thyroxin, in fact, has an added growth-stimulating effect on involuted lymphatic tissue*, as has been shown by the aforementioned studies to apply to thyrotropic hormone The present investigation was made for these purposes

## MATERIAL AND METHODS

The experimental animals were young male guinea pigs weighing 150–250 g [illegible] 21 animals *Group* [illegible]

has been su[illegible] [illegible] cords of the lymphatic tissue (*Gyllensten* [illegible] [illegible]utaneous injection of 10 µg/100 g of body [illegible] [illegible]nan-La Roche) and killed 4 days later [illegible] [illegible]racortenol® intraperitoneally and 4 days *Group* [illegible] later, thyroxin (10 µg/100 g of body weight subcutaneously) and killed after a further 4 days *i e* 8 days after corticosteroid administ[illegible]

The technique used for preparation of the organs and histological examination was the same as the one used in a previous study (*Gyllensten* 1962) The cervical scapular and inguinal lymph nodes were dissected out quantitatively and weighed when fresh as were the thymus spleen and thyroid gland The lymphatic organs [illegible]

lymphocytes transitional cells immature and mature plasma cells The differential counts were made with an immersion optical system at a magnification of 1 000 ×

## RESULTS

The relative weight of the organs (expressed as mg 100 g of body weight) in the various groups is shown in Table 1)

TABLE 1

*Relative Weight of the Organs (mg/100 g of Body Weight) in the Different Groups (for Explanation see Text)*

| Group | Lymph-node tissue Cervical+ Scapular+Inguinal | Thymus | Spleen | Thyroid |
|---|---|---|---|---|
| C | 168 9 | 142 5 | 118 8 | 20 8 |
| U | 149 8 | 106 9 | 115 5 | 18 9 |
| T | 188 1 | 126 8 | 144 6 | 19 8 |
| *UT* | *200 3* | *129 3* | *134 1* | *20 9* |

If the weight of the different organs in the treated animals is compared with the corresponding weight in the controls the following percentage variations are obtained

| Group | Lymph node mass | Thymus | Spleen | Thyroid |
|---|---|---|---|---|
| U | −11 3 % | −25 0 % | −2 8 % | −9 1 % |
| T | +11 4 % | −11 0 % | +21 7 % | −4 8 % |
| UT | +18 6 % | −9 3 % | +12 9 % | +0 5 % |

It is evident from these figures that thyroxin produced an increase in weight of the lymph node tissue, both with an without previous steroid treatment As could be expected administration of corticosteroid alone produced a decrease in weight Subsequent *thyroxin* treatment (group UT) was not only able to *compensate for this steroid* induced involution but also brought about an additional gain in weight so that the combination of steroid *and thyroxin* treatment produced a greater increase in weight than thyroxin alone The changes in weight of the lymph node mass in *relation to that in the controls* are almost significant ($p < 0.05$) whereas the difference between the weight of the lymph node mass in the animals given steroid only and that in the animals given both steroid and thyroxin is significant ($p < 0.01$)

*As far as the* other organs are concerned no significant difference was *present between the* effect of thyroxin with and without previous

*administration of steroid.* The differences between the weights of the organs and those in the controls are not significant, except for the spleen, where an almost significant increase in weight is present in groups T and UT, *i.e.*, a gain in weight after thyroxin treatment. *No differences between the body weights were recorded.*

On the basis of the differential counts, a calculation was made in each specimen of the ratio of the number of special cells of each category (*i.e.*, plasma cells, transitional cells or lymphocytes) to the number of reticular cells. The ratios obtained in this way comprise a measure of the relative composition of the organ in question in relation to the reticular cells, which can be assumed to remain stable in the tissue. Table 2 shows the mean values of these ratios in the cervical lymph nodes of the different groups.

*TABLE 2*

*Ratio of Plasma Cells (pc), Transitional Cells (tc) and Lymphocytes (lc), Respectively to Reticular Cells (rc) in the Different Groups*

| Group | Cervical lymph nodes | | | | |
|---|---|---|---|---|---|
| | Medulla | | | Cortex | |
| | pc/rc | tc/rc | lc/rc | tc/rc | lc/rc |
| *C* | *0.152* | *0.572* | *1.183* | *0.464* | 5.933 |
| U | 0.192 | 0.567 | 1.299 | 0.402 | 5.770 |
| T | 0.340 | 0.705 | 1.212 | 0.489 | 6.018 |
| UT | 0.329 | 0.843 | 1.284 | 0.591 | 5.499 |

It is seen that an increased differentiation of plasma cells, transitional cells, and lymphocytes appeared in the medulla in all groups given hormone treatment. The combination of thyroxin and Ultracortenol potentiated the increase in transitional cells (blast cells), whereas the other, more highly differentiated cells (plasma cells and lymphocytes) were inclined to show less proliferation after combined treatment than after thyroxin alone, or Ultracortenol alone. In the cortex as well, a potentiated stimulation of the transitional cells resulted from a combination of steroid and thyroxin, despite the fact that Ultracortenol alone produced a decrease in number of these cells. The lymphocytes in the cortex also reacted by a decrease—about the same as the lymphocytes in the medulla—to combined treatment with steroid and thyroxin.

In the lymph nodes of the extremities—as in those of the cervical region—the plasma cells *increased in number after administration of* thyroxin only and of steroid only; the increase was smaller when the two treatments were *combined. The changes in composition of the* medullary cords of the lymph nodes in the relevant regions in the treated groups were as follows (the values denote the percentage changes in the mean ratio of plasma cells to reticular cells):

| Group | Lymph nodes Cervical | Scapular | Inguinal |
|---|---|---|---|
| U | +26 6 % | +2 2 % | +7 1 % |
| T | +123 6 % | +67 0 % | +68 7 % |
| UT | +116 4 % | +11 5 % | +45 7 % |

Statistically the increase in plasma cell content is significant in each of the treatment groups as compared to that in the controls The increase in transitional cells in the cervical lymph nodes is also significant In the lymph nodes of the extremities on the contrary the increase is not significant this also applies to changes in the lymphocyte content Calculated on all the experimental groups and the combined lymph nodes the decrease in plasma cell stimulating effect in combined treatment as compared to that with corticosteroid alone or with thyroxin alone is almost significant ($p < 0\ 05$)

No significant change could be demonstrated in the ratio of mature to immature plasma cells

In the spleen a highly significant increase in the number of plasma cells and transitional cells took place after thyroxin treatment Conversely treatment with Ultracortenol alone or in combination with thyroxin did not produce any significant change in the number of these cells Nor was any significant difference present in the lymphocyte content of the spleen after any form of treatment in question

## DISCUSSION

The present investigation confirms the observation that exogenous thyroxin is able also to produce a relative greater increase in the weight of lymphatic tissue after involution than that in normal lymphatic tissue The results therefore are in agreement with those of earlier studies showing that *endogenous* thyroxin has a similar effect after stimulation of the thyroid gland with thyrotropic hormone (*Barnholdt von Euler et al*) Consequently the effect must be bound to the thyroxin and not to the thyrotropin nor to any other hypophyseal factor which possibly might be supplied together with the thyrotropin

As far as the cellular changes during stimulated regeneration are concerned the action of thyroxin on the involuted lymph node tissue differed from that on normal lymph node tissue Earlier studies as well as the present investigation have shown that thyroxin stimulates *both* maturation of lymphatic cells—mainly pyroninophil cells—and proliferation of more immature cell forms (transitional cells) In the involuted lymph node tissue the regeneration of which was markedly stimulated by thyroxin this hormone had a greater effect on the growth processes i e on the increase in number of immature cells than on the maturation processes The spleen on the contrary the regeneration of which did not seem to be stimulated by thyroxin administration showed no such signs of a modified reaction to thyroxin when it was given after previous corticosteroid administration

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| Group | Cervical | Lymph nodes Scapular | Inguinal |
|---|---|---|---|
| U | +26.6 % | +2.2 % | +7.1 % |
| T | +103.6 % | +67.0 % | +68.7 % |
| UT | +116.4 % | +11.5 % | +45.7 % |

Statistically the increase in plasma cell content is significant in each of the treatment groups as compared to that in the controls. The increase in transitional cells in the cervical lymph nodes is also significant. In the lymph nodes of the extremities, on the contrary, the increase is not significant; this also applies to changes in the lymphocyte content. Calculated on all the experimental groups and the combined lymph nodes, the decrease in plasma cell stimulating effect in combined treatment as compared to that with corticosteroid alone or with thyroxin alone is almost significant ($p < 0.05$).

No significant change could be demonstrated in the ratio of mature to immature plasma cells.

In the spleen, a highly significant increase in the number of plasma cells and transitional cells took place after thyroxin treatment. Conversely, treatment with Ultracortenol alone or in combination with thyroxin did not produce any significant change in the number of these cells. Nor was any significant difference present in the lymphocyte content of the spleen after any form of treatment in question.

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As in earlier studies, some parallelism was evident between the changes in weight of the thyroid gland and those of the thymo lym phatic tissue (for discussion, see *Gyllensten* 1953, 1962) This may be associated possibly in some way with the previously discussed homeo stasis which has been found to exist between the thyroid gland and the thymo-lymphatic tissue, during growth and regeneration of the latter

## SUMMARY

Exogenous thyroxin is found to stimulate regeneration in the lymph node tissue of young guinea-pigs, after thymo-lymphatic involution induced by administration of a glycocorticosteroid Differential counts in sections of lymphatic tissue show that thyroxin stimulates both *differentiation* (maturation) and *proliferation* in the tissue, judged by the relative number of mature cells (plasma cells and lymphocytes) as compared to immature forms (transitional cells and reticular cells) A comparison between the effect of thyroxin on involuted lymph node tissue and on normal such tissue shows that, in the former case, the hormone produces greater stimulation of growth and less stimulation of maturation than in lymphatic tissue which has not undergone invo lution by previous administration of corticosteroid

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# THE CELLULAR COMPOSITION OF LYMPHATIC TISSUE DURING INVOLUTION AFTER ADMINISTRATION OF CORTICOSTEROID

*A Quantitative Study in Guinea-Pigs*

*By*

LARS GYLLENSTEN

Received 19 iii 62

Involution of the thymus and lymphatic tissue is one of the most characteristic reactions in the stress syndrome, in other activation of the endogenous secretion of the adrenal cortex, and after administration of certain steroids, particularly glycocorticosteroids (*Selye* 1936, *Dougherty* 1952, 1959, *Ehrlich & Seifter* 1953, *Kass et al* 1953, *Harris et al* 1954)

The histological changes in steroid induced involution in various animal species have been studied by a number of authors, with divergent results in certain respects *Nuclear pyknosis* and *lymphocytolysis*, most pronounced in the thymic cortex, have been observed during the first hours after administration of adrenocorticotropic hormone or corticosteroids (*Dougherty & White* 1944, 1945, 1946, *Baker et al* 1951, *Ringertz et al* 1952 *Ehrlich & Seifter* 1953 *Hill & Popisil* 1960) This degenerative phase has also been stated to be accompanied by a decrease in the frequency of mitosis in thymus and/or lymphatic tissue (*Dougherty & White* 1945, *Pfeiffer et al* 1952, *Robbins et al* 1955, *Dougherty* 1959) Moreover, studies on other tissues seem to indicate that certain steroids have a mitosis-inhibiting effect (*Bullough* 1951/2, *Fautrez et al* 1960, *Gelfant* 1960, *Wellington & Moon* 1961), or cause inhibition of protein or nucleoprotein synthesis (*Clark & Stoorck* 1946, *Engel* 1951, *Hall & White* 1952, *Kit et al* 1953, 1954, *Blecher & White* 1958)

The action of steroids on the plasma cells has aroused special attention because of their ability, under certain conditions, to reduce the production or concentration of antibodies (*Schwartzman* 1953, *Kass et al* 1953, *Berglund* 1956, *McMaster* 1961) Some ... have re- ... lympho- ... an in-

The expenses of this investigation were partly defrayed by a grant from the Swedish Cancer Society

crease in the plasma cells under the influence of steroids (*Dougherty & White* 1946, *Ringertz et al* 1953, *Lundin et al* 1954, *Hill & Popsid* 1960) Finally, the number of plasma cells has also been reported to be uninfluenced by administration of steroids (*Baker et al* 1951)

In view of the conflicting statements on *e g* the reaction of the plasma cells in lymphatic tissue on steroid-induced involution, I considered it warranted to make a quantitative study, also intended to serve as a basis for further investigation of the regeneration phenomenon in lymphatic tissue after involution Quantitative analyses of this kind, with differential counts in sectioned material, have been made earlier by *Kindred* (1938, 1955) and *Ringertz & Adamson* (1950), among others A special method was previously devised for quantitative studies of the plasma cells during postnatal development, as well as in thyroxin-induced lymphatic hyperplasia (*Carlsson & Gyllensten* 1958, *Ernstrom & Gyllensten* 1959), and has been applied in the present investigation

## MATERIAL AND METHODS

Young male guinea pigs weighing 150–250 g were used The controls consisted of 21 untreated guinea pigs Altogether 65 animals were given 10 mg of prednisolone trimethyl acetate (Ultracortenol® Ciba) intraperitoneally and were killed by a blow on the neck 1 2, 4 8 or 16 days later, in groups of 13 on each occasion The cervical scapular and inguinal lymph nodes were [illegible] and weighed in the fresh state each group separatel[illegible] earlier (*Gyllensten* 1951) The thymus spleen and [illegible] out and weighed A good correlation has been found between the weight of the prepared lymph nodes and the total mass of lymph node tissue in the guinea pigs (*Gyllensten* 1953)

The lymph nodes and spleen were fixed according to Carnoy cut into sections 5 µ thick after embedding in paraffin and stained with methyl green pyronine by a [illegible] 1958) A differential count was made in [illegible] magnification of 1 000 × [illegible] dulla respectively of the lymph nodes and the same number in the pulp of the spleen without division into white and red pulp The cells were taken at random *i e* those cells were counted whose nucleus was visible under a reference line in the eyepiece The differential count did not include vessels connective tissue septa or the reaction centres of the lymph nodes

The cells were classified according to the same criteria as those used in earlier studies (*Carlsson & Gyllensten* 1958 *Ernstrom & Gyllensten* 1959) which correspond to *Fagraeus* classification of 1948 The following five groups of cells were distinguished reticular cells lymphocytes transitional cells immature and mature plasma cells The last three categories are strongly pyroninophil Although the terminology used is not that recommended in Prague (*Fagraeus* 1960) it is motivated by the desirability of conformity with the aforementioned earlier studies

Occasional polymorphonuclear leukocytes and other unclassified cells were not included in the count

## RESULTS

The relative weight of the organs in the 21 controls is shown in Table 1

The values in the prednisolone treated animals (13 in each group), expressed as a percentage of the corresponding values in the controls, are listed in Table 2

TABLE 1

*Relative Weight of the Organs (mg/100 g of Body Weight) in the 21 Controls*

| Lymph nodes | | | Total lymph node tissue | Thymus | Spleen | Thyroid |
|---|---|---|---|---|---|---|
| Cervical | Scapular | Inguinal | | | | |
| 109.90 | 30.36 | 28.60 | 168.85 | 142.53 | 118.83 | 20.77 |

TABLE 2

*Weight of the Organs in the Prednisolone Treated Animals (13 per group) in per Cent of the Corresponding Values in the Controls*

| Day after treatment | Lymph nodes | | | Total lymph node tissue | Thymus | Spleen | Thyroid |
|---|---|---|---|---|---|---|---|
| | Cervical | Scapular | Inguinal | | | | |
| 1 | 83.0 | 98.3 | 101.7 | 88.6 | 95.0 | 96.1 | 96.9 |
| 2 | 97.0 | 97.6 | 99.6 | 97.6 | 103.7 | 105.6 | 99.2 |
| 4 | 86.7 | 71.3 | 87.2 | 84.0 | 87.4 | 97.4 | 90.8 |
| 8 | 94.8 | 75.0 | 81.0 | 88.9 | 76.0 | 96.2 | 88.2 |
| 16 | 103.0 | 87.3 | 91.5 | 98.2 | 104.8 | 109.9 | 94.2 |

It is seen that a *decrease in weight* of the total *lymph-node tissue* occurred, it was maximal on day 4, after which the weight rose towards the normal value on day 16. On days 4 and 8, the decrease is highly significant ($p < 0.001$). The regenerative increase in weight was slightly slower in the lymph nodes of the extremities than in those of the cervical region. This is in conformity with earlier observations on functional differences between different regional groups of lymph nodes (*Gyllensten* 1950, *Carlsson & Gyllensten* 1958, *Ernstrom & Gyllensten* 1959).

The *thymus* showed a somewhat greater, also highly significant decrease in weight, most marked on day 8. The decrease in weight of the *thyroid gland* as well is highly significant, whereas the changes in weight of the *spleen* are not significant.

The *percentage distribution* of the various cell types in the *lymph-node tissue* and *spleen* of the controls is seen in Table 3.

TABLE 3

*Percentage Cellular Composition of the Lymph Node Tissue and Spleen in the 21 Controls*

| Organ | Plasma cells (mature + immature) | Transitional cells | Reticular cells | Lymphocytes |
|---|---|---|---|---|
| Lymph node cortex | 0.05 | 6.25 | 14.18 | 79.51 |
| Lymph node medulla | | | | |
| Cervical | 4.77 | 17.25 | 37.54 | 40.47 |
| Scapular | 2.43 | 12.76 | 44.01 | 40.82 |
| Inguinal | 1.34 | 15.60 | 40.69 | 42.38 |
| Spleen | 0.51 | 14.81 | 65.61 | 19.07 |

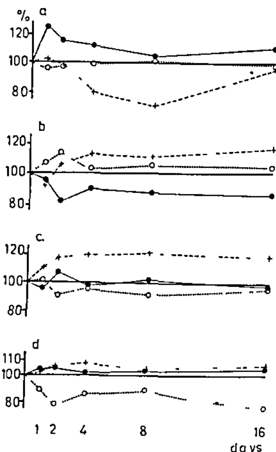



*Fig 1*

Changes in the cellular composition of the lymphatic tissue 1 16 days after injection of glycocorticosteroid as compared to that in the controls (=100%) *a* = lymph node cortex, *b* = medullary cords of cervical lymph nodes, *c* = medullary cords of lymph nodes in the extremities, *d* = spleen

—●— reticular cells

– + – – pyroninophil cells (transitional cells + immature and mature plasma cells)

○ lymphocytes

Of the plasma cells in the medullary cords of the cervical lymph nodes, 20 per cent were mature and the rest immature, in the lymph nodes of the extremities, 16 and 18 per cent, respectively, were mature The number of plasma cells in the other organs was so small that such a classification was not merited

The differential composition of the corresponding tissues in the experimental animals, as compared to that in the controls, is apparent from Fig 1 Since no significant difference was present in this respect between the scapular and inguinal lymph nodes, their values have been combined

It is evident from Fig 1 that the *lymph-node cortex* showed a marked, relative decrease in pyroninophil cells (almost exclusively transitional cells) Histologically, nuclear pyknosis could be observed in certain lymphocytes in the cortex, particularly on days 1 and 2 The incidence

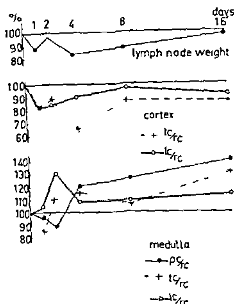



*Fig 2*

Changes in weight and relative cellular composition of cervical lymph nodes 1 16 days after injection of corticosteroid The changes are given as percentage deviations from corresponding control values The cellular composition of the cortex and medulla respectively is calculated as the relation between the number of plasma cells (*pc*) transitional cells (*tc*) and lymphocytes (*lc*) respectively and the number of reticular cells (*rc*) the reticular cells thus being taken as reference cells

of pyknotic cells however, was extremely low (less than 1 per cent), consequently, atrophy of the cortex cannot be explained by such lymphocytolysis as could be demonstrated in the form of pyknosis The lymphocytes nevertheless had decreased in number as compared to the reticular cells This decrease in lymphocytes, as well as in transitional cells in relation to reticular cells can be inferred from Fig 2, and showed some correlation to the decrease in weight

The relative decrease in both lymphocytes and pyroninophil cells in the cortex is highly significant ($p < 0.001$)

In the *medullary cords of the cervical lymph nodes*, there was an initial relative decrease in the pyroninophil cells on day 1, and in the plasma cells on day 2 as well (Figs 1 and 2) The decrease was followed by a relative increase in the cell types in question during days 4 16 The relative lymphocyte content showed an increase during the whole experimental period The increase in relative lymphocyte content and in the content of pyroninophil cells after day 2 is highly significant The initial decrease in pyroninophil cells is almost significant ($p < 0.05$)

In the *medullary cords of the lymph nodes of the extremities*, no initial decrease occurred in the number of pyroninophil cells, there was,

on the contrary, a significant increase during the whole experimental period Although *a slight relative decrease in lymphocytes was ob*served, it was not significant

In the *spleen,* a slight but not significant relative increase could be demonstrated in the number of pyroninophil cells, as well as a significant relative decrease in lymphocytes

A comparison between the reactions in the various pyroninophil cells showed a significant increase (+ 19 4 per cent) in the plasma cells, as compared to the transitional cells, in the medullary cords of the cervical lymph nodes, and an *insignificant increase in the medulla* of the other lymph nodes This increase in maturation reached a maximum on day 8 in the cervical lymph nodes, and on day 16 in the lymph nodes *of the extremities, and appeared from the start of the experimental* period A comparison between mature and immature plasma cells also showed stimulated maturation of the pyroninophil cells under the influence of the corticosteroid, expressed as an increase in the number of mature plasma cells in relation to immature This increased maturation of plasma cells was highly significant in the cervical lymph nodes, and took place during the whole experimental period, with a maximum on day 4 In the lymph nodes of the extremities, such maturation was barely perceptible, and thus not significant

In view of the low content of plasma cells in the lymph-node cortex and the spleen, no statistical comparison could be made between possible *changes in their degree of maturation*

## DISCUSSION

During corticosteroid-induced involution, the reactions in the cortex of the lymph nodes are, on the whole, the reverse of those in the medullary cords Thus, in the cortex, a relative decrease occurs in the number of lymphocytes and transitional cells as compared to reticular cells, *i e*, a "de differentiation" This decrease is largely correlated to the decrease in weight, with a maximum on day 4, and explains the decrease in weight, since the cortex is responsible for the greater part of the weight of the lymph nodes

In the medullary cords, on the contrary, a relative increase in number of pyroninophil cells—and, to some extent, also of lymphocytes—takes *place during the greater part of the experimental period, i e*, a "differentiation These changes can be interpreted as signs of regeneration in the lymph nodes, originating in the medulla This regeneration starts *even before involution of the cortex has reached a maximum* A description of such function in the lymph node marrow has been given earlier by *Ringertz et al* (1951)

Certain regional *differences between lymph node groups* were observed in the present study, in conformity with those described previously by *Gyllensten* and co-workers (1950, 1958, 1959) and—as far as

the differential composition of various lymph nodes is concerned—also by *Fujii* (1960) The cervical lymph nodes gave evidence of being more highly differentiated with respect to the content of highly differentiated cells (plasma cells and lymphocytes) in the medullary cords The changes after steroid administration were also more distinct in this region, and had a higher degree of significance than those in the lymph nodes of the extremities Moreover, an initial decrease occurred in the number of pyroninophil cells, reminiscent of the conditions in the lymph-node cortex, and indicating that steroid-induced involution is, at the start, an expression of a generalized reaction in the lymphatic system

The changes in the spleen resembled those in the lymph nodes, consisting of an inappreciable increase in pyroninophil cells (localized to the red pulp), and a decrease in lymphocytes (localized to the white pulp) The spleen thus seems to combine the reactions characteristic of both the cortex and the medulla of the lymph nodes, *i e*, atrophy and regeneration, respectively This may possibly explain the less marked decrease in weight of the spleen

On day 2, a consistent relative increase in weight was recorded in all lymphatic organs as compared to day 1, which produced a break in the involution curve This might have been due to fluid retention and oedema (*Hill & Popisil* 1960)

As mentioned in the introduction to this paper, certain investigations indicate that glycocorticosteroids can produce inhibition of mitosis This could partly explain the decrease in weight of the lymphatic tissue, as well as the relative preponderance of mature cells as compared to immature cells in the medullary cords Furthermore, the time course of involution, with a maximum several days after administration of the steroid, when it has long since been inactivated (*Dougherty* 1959), can be explained partly by an inhibition of mitosis, involving chiefly the earliest stages of lymphocytopoiesis and plasmocytopoiesis

The comparatively marked increase in pyroninophil cells—greatest in the more mature forms—in the medullary cords can scarcely be ascribed only to inhibition of mitosis in immature cells, with a resulting relative decrease in their number This is because the increase in the mature forms of cells persists, without diminishing, during the whole experimental period, this applies even during the [illegible]

It has been claimed that an increase in mature or old cells in a tissue is able to stimulate mitotic activity, whereas a decrease in their number can inhibit this activity (*Teir & Ravanti* 1953, *Osgood* 1959, *Teir & Lahtiharju* 1961) Such a homeostatic mechanism can be envisaged to underlie regeneration taking place in the medullary cords of the lymph nodes after steroid induced involution Thus, the relative

increase in mature cells (plasma cells and lymphocytes), as well as possible early lysis of such cells, would be able to stimulate mitotic activity in the medullary cords, with a resulting migration of newly formed cells into the cortex and/or circulation The more intense migration of the lymphocytes—particularly into the circulation—can explain the differences that have been observed between the two forms of cells under the influence of steroids

Certain investigations seem to show that the incidence of lymphatic tumours may be raised after administration of steroids (*Gardner et al* 1944, *Toch et al* 1956, *Mühlbock & Boot* 1959), although the relevant data are conflicting (*Kaplan et al* 1954) An increased new formation of cells in the medulla of the lymph nodes during and after steroid induced involution might, in fact, imply a greater sensitivity to cancerogenic injuries, *e g* roentgen irradiation

The increase in plasma cells during steroid-induced involution appears to contradict the results of studies showing that steroids, under certain conditions, can inhibit the formation of antibodies (for references, see Introduction) The contradiction, however, is merely apparent if—as stated by *Fagraeus* (1948), among others—it is the immature pyroninophil cells that are responsible for antibody formation In fact, the differential counts show a preponderance of mature plasma cells under the influence of steroids, and this "hyper-maturity" might well be envisaged to disturb the ability of the cells to produce antibodies

In the present investigation, a certain parallelism was observed between lymphatic involution and thyroid decrease, with a rise in weight of the thyroid gland in connexion with regeneration of the involuted lymphatic tissue This might be an indication of the homeostatic balance which, to some extent, exists between thyroid and thymo-lymphatic tissue (*Gyllensten* 1953, *Barnholdt-von Euler et al* 1959)

## SUMMARY


1 The present investigation is a quantitative one Differential cell counts are made in sections of the lymph-node tissue and spleen of guinea-pigs, during involution and regeneration after a single, intraperitoneal injection of glycocorticosteroid Regeneration is largely completed 16 days after steroid administration

2 In the lymph-node cortex, a decrease is found in the number of lymphocytes and pyroninophil cells, in good correlation to the decrease in weight As a result of this decrease in highly differentiated cells, a relative increase takes place in the reticular cells, *i e*, a "de-differentiation"

3 In the medullary cords of the lymph nodes, a highly significant, relative increase in the number of pyroninophil cells occurs during most of the experimental period, as well as a greater degree of maturation of

such cells with the appearance of a larger number of mature plasma cells i e a d fferentiation

4 Certain regional differences are observed l etween lymph node groups The cervical lymph nodes have a higher content of pyroninophil cells in their medullary cords Here there is also a decrease in number of plasma cells during the first 2 days after administration of the steroid and on the first day of immature pyroninophil cells as well After this initial reaction follows the aforementioned conspicuous in crease of the same cells during the rest of the experimental period An increased differentiation is also observed with respect to the lym phocytes which increase in number during the whole experimental perio l The lymph nodes of the extremities do not exhibit these changes

5 In the spleen s milar but less pronounced changes take place as in the lymph nodes in the form of a relative increase in the pyroninophil cells and a decrease in the number of lymphocytes

6 The mechanism underlying steroid induced involution is dis cussed The probability is suggested of a homeostatic balance with involution in the cortex of the lymph nodes and regeneration with an early onset in the medulla The effect of steroids on the lymphatic tissue can possibly be exl lained as a combination of cytolysis with an early onset inhibition of mitosis chiefly in immature cells and in creased differentiation of more mature cells Accumulation of mature or hyl er mature and damaged cells may have a mitosis stimulating action and thus secondarily elicit proliferation in more immature forms of cells with regeneration as a result

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*Klijn* (8) reported no such finding Most methods recommend 1 to 7 day old sheep cells (*1 18*) *Dempster* (*4*) *however noted that the* antibody agglutinated fresh erythrocytes to the same titre as erythro cytes which had been kept for one day According to this author the keeping of the cells for one day is only likely to increase the agglutina tion effect produced by the normal Forssman antibody

Both the concentration of cell suspension (1 17) and the tempera ture of incubation definitely affect the final result (17) Cold aggluti nins present in the serum are particularly likely to lead to error (22) if test tubes are kept overnight in a refrigerator this possibility of error from cold agglutinins can be eliminated by re heating tubes at 37° C (17 22) Ice box incubation only incurs the nonspecific action of cold agglutinins Although higher titres are obtained in this way neither the sensitivity nor the specificity of the test is increased (3) Incubation at 37° C therefore is alone to be recommended (4 18)

To evaluate the importance of absorption tests in the serological diagnosis of infectious mononucleosis and to give an idea of the in cidence of infectious mononucleosis in Southwest Finland the Paul Bunnell material of Turku University Department of Medical Micro biology over the last 8 years is reported on below

## MATERIAL AND METHOD

The material comprises the 1963 blood specimens of patients suspected of having infectious mononucleosis tested by the Turku University Department of Medical Microbiology in 1954 1961

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w [illegible] and the following morning the result was checked by the naked eye as the tubes were gently shaken The degree of agglutina tion was assessed as + [illegible] +++ One tube of the series provided cell control with [illegible]

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*FROM THE DEPARTMENT OF MEDICAL MICROBIOLOGY, UNIVERSITY OF TURKU, FINLAND (HEAD PROFESSOR EERO MUSTAKALLIO M.D.)*

# DIFFERENTIAL ABSORPTION TESTS IN THE DIAGNOSIS OF INFECTIOUS MONONUCLEOSIS

*By*

SIMO VIRTANEN

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The Paul-Bunnell test or heterophilic antibody test is non-specific (3, 14). Its exact nature is still obscure, but it may be similar to e.g. the Wassermann reaction in the diagnosis of syphilis, or the Weil-Felix reaction in some of the rickettsial diseases (13).

Except in infectious mononucleosis, relatively high titres of sheep red-cell agglutinins may occur in normal subjects after an injection of horse serum, or after various known and unknown antigen stimuli (2). Elevated titre values are noted in many diseases of the lymphatic system (15).

The antibody to infectious mononucleosis can be distinguished by absorption tests from the other factors agglutinating the red blood cells of sheep. The sheep cell agglutinins in infectious mononucleosis can be completely absorbed by beef erythrocytes but are not reduced significantly by absorption with guinea pig kidney. In serum sickness sheep cell agglutinins can be completely absorbed by both guinea-pig kidney and beef erythrocytes. Sheep cell agglutinins in normal human sera are not absorbed by beef cells whereas they are by guinea-pig kidney (10, 14, 18).

Differential absorption test is highly specific for infectious mononucleosis (3, 9): a titre ≧ 1:10 after absorption with guinea pig kidney is valid for its diagnosis (13, 14). According to *Leibowitz* (10) and *Klyn* (8), a significant titre is one which fulfills the absorption characteristics of infectious mononucleosis, whether the titre be high or low.

Contradictory results in the Paul-Bunnell test are usually attributable to differences in the methods employed (17, 22). It is absolutely necessary to indicate the titre in the form of the final dilution of the serum after the addition of cells (17, 18).

Information about the influence on results of the sheep cells used in titration is contradictory. According to *Keiper* (7) and *Zarafonetis et al.* (21), the cells of different sheep give different titres, whereas

1 Aided by a grant from the *Rockefeller Foundation*

test titres of 1/10 1/40, only four (3.3 per cent) contained infectious mononucleosis antibodies. They account for 6.3 per cent of the infectious mononucleosis sera in the present material.

The incidence of positive sera by year is shown in Table 3. Of the total material 11.3 per cent showed positive sera. The annual incidence of positive cases is shown in Table 4.

TABLE 3

*The Annual Occurrence of Sera with Absorption Pattern Typical of Infectious Mononucleosis*

| Year | Infectious mononucleosis sera | | Negative sera | Total |
|---|---|---|---|---|
| | Number | Per cent | | |
| 1954 | 19 | 9.2 | 187 | 206 |
| 1955 | 26 | 8.9 | 265 | 291 |
| 1956 | 20 | 6.9 | 270 | 290 |
| 1957 | 25 | 9.5 | 239 | 264 |
| 1958 | 23 | 12.5 | 161 | 184 |
| 1959 | 27 | 13.2 | 177 | 204 |
| 1960 | 37 | 16.2 | 192 | 229 |
| 1961 | 45 | 15.3 | 250 | 295 |
| | 222 | 11.3 | 1741 | 1963 |

TABLE 4

*Incidence of Serologically Verified Cases of Infectious Mononucleosis by Year*

| Year | Number of cases |
|---|---|
| 1954 | 17 |
| 1955 | 23 |
| 1956 | 20 |
| 1957 | 26 |
| 1958 | 24 |
| 1959 | 27 |
| 1960 | 29 |
| 1961 | 39 |
| | 205 |

## DISCUSSION

The etiological agent of infectious mononucleosis has not as yet been detected (14). Experimental transmission, apart from a few suggestive attempts, has not been confirmed, while attempts to transmit the infection to experimental animals have also failed or have yielded no conclusive results (6, 14).

Infectious mononucleosis usually occurs in sporadic cases (5), and cross infection in hospital wards or between room mates is almost unknown (5, 6). Reports on epidemics of infectious mononucleosis are generally not conclusive to the critical observer (6). In cases where

which the haemagglutination test was performed in the usual way with the serial dilution 1/8 to 1/4096

*For Method II*, 20 per cent guinea pig kidney emulsion in saline and 20 per cent *beef red cell* suspension in saline were prepared according to *Mackie & McCartney* (12) 1 25 ml guinea pig kidney emulsion was added to 0 25 ml of serum and 1 25 ml *of beef cell* suspension to another 0 25 ml batch The tubes were left at room temperature for 1 hour and centrifuged, after which the haemagglutination test was performed in the manner described under Method II The serial dilution in this case was 1/10 to 1/10240

## RESULTS

The results of the Paul-Bunnell tests performed according to Method I are given in Table 1 Absorption results typical of infectious mononucleosis was obtained in 154 cases, 10 1 per cent Of the sera whose titre in the presumptive test was ≧ 1/64, 65 (29 7 per cent) became negative after guinea-pig kidney absorption In 8 cases the serum did not suffice for absorption, in 6 of these cases the titre was 1/64, in one, 1/128 and in one, 1/256

TABLE 1
*Absorption with Guinea Pig Kidney (Material I)*

| Titre in presumptive test | Treated with guinea pig kidney | | Untreated | Total number |
|---|---|---|---|---|
| | Not absorbed = antibody of infectious mononucleosis | Absorbed | | |
| ≧ 1/64 | 154 | 65 | 8 | 227 |
| < 1/64 | | – | 1304 | 1304 |
| | 154 | 65 | 1312 | 1531 |

TABLE 2
*Absorption with Guinea Pig Kidney and with Beef Cells (Material II)*

| Titre in presumptive test | Treated with guinea pig kidney | | Treated with beef cells | | Untreated | Total number |
|---|---|---|---|---|---|---|
| | Not absorbed — antibody *mononucleosis of infectious* | Absorbed | *Not absorbed* | Absorbed | | |
| ≧ 1/80 | 64 | 19 | 0 | 83 | 2 | 85 |
| 1/10 1/40 | 4 | 119 | 0 | 123 | | 123 |
| < 1/10 | | | | | 224 | 224 |
| | 68 | 138 | 0 | 206 | 226 | 432 |

The results of the determinations according to Method II are given in Table 2 Of the sera tested, 68 (16 1 per cent) were typical infectious mononucleosis sera Nearly half (47 8 per cent) of the sera were absorbed both with guinea-pig kidney and beef cells In two cases there was insufficient serum for absorption The titres of these sera in the presumptive test were 1/80 and 1/320 Of 122 sera with presumptive

est titres of 1/10–1/40, only four (3.3 per cent) contained infectious mononucleosis antibodies. They account for 6.3 per cent of the infectious mononucleosis sera in the present material.

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## RESULTS

The results of the Paul-Bunnell tests performed according to Method I are given in Table 1. Absorption results typical of infectious mononucleosis was obtained in 154 cases, 10.1 per cent. Of the sera whose titre in the presumptive test was ≧ 1/64, 65 (29.7 per cent) became negative after guinea-pig kidney absorption. In 8 cases the serum did not suffice for absorption, in 6 of these cases the titre was 1/64, in one, 1/128 and in one, 1/256.

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*Absorption with Guinea Pig Kidney and with Beef Cells (Material II)*

| Titre in presumptive test | Treated with guinea pig kidney | | Treated with beef cells | | Untreated | Total number |
|---|---|---|---|---|---|---|
| | Not absorbed antibody of infectious mononucleosis | Absorbed | Not absorbed | Absorbed | | |
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| 1/10–1/40 | 4 | 119 | 0 | 123 | | 123 |
| < 1/10 | | | | – | 224 | 224 |
| | 68 | 138 | 0 | 206 | 226 | 432 |

The results of the determinations according to Method II are given in Table 2. Of the sera tested, 68 (16.1 per cent) were typical infectious mononucleosis sera. Nearly half (47.8 per cent) of the sera were absorbed both with guinea-pig kidney and beef cells. In two cases there was insufficient serum for absorption. The titres of these sera in the presumptive test were 1/80 and 1/320. Of 122 sera with presumptive

shows a slight increase, though this is statistically significant only for 1961 ($P < 0.05$). This may be connected with the change in determination technique.

The absorption of low titre sera involves a great deal of additional work. For determinations according to Method II, 47.8 per cent of the specimens had to be absorbed, whereas in determinations according to Method I with sera of titres ≧ 1/64 only absorbed the proportion of absorbed samples was no more than 14.3 per cent. Had absorption not been carried out, 4 (6.3 per cent) of the 68 positive samples of Material II would have remained undetected. Absorption is of even greater importance in *the elimination of false positive results*. *In* 29.7 per cent of the sera of Material I with titres ≧ 1/64 in the presumptive test no antibody to infectious mononucleosis was involved. Of the Material II sera with titres ≧ 1/80 in the presumptive test, 22.9 per cent had no antibodies to mononucleosis.

SUMMARY

Sheep red cell agglutinins of 1,963 serum samples from patients with suspected infectious mononucleosis were tested. In 425 cases the test was complemented by absorption procedures: in 219 cases with guinea pig kidney absorption and 206 cases with both guinea pig kidney and beef cell absorption. Of the sera tested 11.3 per cent gave an absorption pattern typical of infectious mononucleosis. The importance of serological reactions in the diagnosis of infectious mononucleosis is discussed. Absorption procedures are declared necessary to confirm the diagnosis. By absorbing also the low-titre sera (1/10–1/40), the number of positive results could be increased by 6.3 per cent.

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absorption tests have been carried out in connection with an epidemic, results have been inconsistent (19, 20) *Shubert et al* (16) describe an epidemic variant of infectious mononucleosis for which the serologic tests were negative They claim that this is a form closely allied to yet different from, the sporadicallly occurring *infectious mononucleosis* According to *Leibowitz* (11), two different but related viruses exist one associated *with sero-positive cases, the other with sero negative* cases Epidemic variety is most often described as the type in which *the heterophil antibody test* is not positive

It is quite possible that a clinical picture resembling that of infectious mononucleosis embraces a number of different entities, some of them ill-defined (6, 14)

Different writers report 83–100 per cent positive Paul-Bunnell tests performed with the absorption technique, in mononucleosis materials Poorer correlations have also been reported, *e g Dempster* (4), with 66 per cent This naturally depends upon the criteria on which the diagnosis of mononucleosis is based The diagnosis of infectious mono nucleosis *should* rest on a *triangular base typical symptomology*, hae matology and serology (5) The first two are far from specific (5, 10) *The sheep-cell agglutination test, complemented by the* absorption technique, is the most specific of the three (5, 10) This test may be used *as an almost absolute guide to the diagnosis of infectious mono* nucleosis (10) It would therefore be wise to regard a positive sheep cell agglutination test as essential *to the* diagnosis of *infectious mono*nucleosis (5)

If the serological tests are correctly performed they are nearly 100 per cent positive in infectious mononucleosis The most frequent causative factors of sero-negativity are 1 failure to perform agglutination tests early enough in the illness 2 failure to perform agglutination tests late enough in the illness Unless the tests are repeated up to three months from the onset of the illness, the case cannot be considered one of sero-negative infectious mononucleosis 3 Failure to employ confirmatory absorption tests on sera with low titres 4 Failure to exclude cases of a confusingly similar clinical picture which are not infectious mononucleosis (10) If the above conditions are met the causative factor may be that 5 different but related viruses may be involved or 6 individuals may occasionally be serologically "inert", meaning that they fail to react to an antigen with the usual production of antibodies (11) If these criteria are applied, the proportion of sero-negative cases in Leibowitz's material is 5 5 per cent (11) Validity of diagnosis of infectious mononucleosis in sero-negative cases will, however, remain somewhat uncertain, at least until the etiological agent is identified and an unquestionable diagnostic test devised (11)

The present material, see Tables 3 and 4, showed a roughly uniform *annual incidence* of infectious mononucleosis This concurs with the sporadic nature of the disease In recent years the number of cases

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# INCIDENCE OF INFECTIOUS MONONUCLEOSIS ANTIBODIES IN BLOOD DONORS

*By*

SIMO VIRTANEN

Received 20 ii 62

The infectious mononucleosis antibody may be distinguished from the other heterophilic antibodies by absorbing the serum with guinea pig kidney and beef red cells (12, 15, 16). This differential absorption test is highly specific for infectious mononucleosis (5). Indeed, it is the most specific of all diagnostic characteristics of infectious mononucleosis (4, 7), and is positive in nearly 100 per cent of the cases (12, 13).

The positive reaction, however, may be extremely transient (3, 7, 15). In *Hoagland's* material (7) the reaction often returned to negative within the first month and usually within 3 months. In *Leibowitz's* material [illegible] third month but no lo[illegible] the reaction usually r[illegible] may remain so for 9[illegible] is somewhat contradictory, and it is obvious that in any given case there is no way of predicting at what levels or for how long the antibodies will persist (2).

An average of 25–26 cases of infectious mononucleosis per year were serologically verified by the Department of Medical Microbiology, University of Turku, in 1954–1961. Since the disease usually takes a benign course and its symptoms are often indefinite, patients rarely consult a doctor. Many of the cases are evidently treated at Outpatient Clinics as "sore throat", angina or pharyngitis without a correct diagnosis. The disease may, moreover, take a completely asymptomatic form (9, 11) and it is therefore evident that the diagnosed cases do not provide an accurate picture of the incidence of the disease. To discover the extent to which the infectious mononucleosis antibody is encountered among the healthy adult population of Finland, the following investigation on blood donors was carried out in the autumn of 1960.

[1] Aided by a grant from the *Rockefeller Foundation*.

12 *Mackie T J & McCartney J E* Handbook of practical bacteriology 9th edition E & S Livingstone Ltd Edinburgh and London 1953
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21 *Zarafonetis C J D & Oster H L* Heterophile agglutination variability of erythrocytes from different sheep J Lab & Clin Med *36* 283 1950
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In another case (titre 1/320) the blood donor had had fever and a sore throat for three days one month earlier, but he had not consulted the doctor or had any treatment. No information was obtainable on the third case (titre 1/20).

## DISCUSSION

Incidence of infectious mononucleosis antibody must be considered indicative of a relatively recent infection. The presence of antibody in about 1 per cent of healthy subjects suggests that the true incidence of the disease is considerably higher than the total of definitely diagnosed cases, which, apart from the three cases listed above, numbered only 29 in 1960. The Turku University Department of Medical Microbiology is the serological laboratory serving a district with a total population of about 650,000. A fairly high incidence of infectious mononucleosis is also suggested by *Klemola's* (10) observations from Helsinki Municipal Epidemic Hospital where 9 cases of infectious mononucleosis were recorded per 100 cases of angina. In Denmark, according to *Thomsen* (17), there were 7.8 cases of mononucleosis per 100 cases of angina. Evidently, therefore, quite a considerable proportion of the cases of infectious mononucleosis escape detection. The reason is probably the same as in the present material: the patients do not consult the doctor at all, and a number of cases are incorrectly diagnosed as angina or "sore throat". In England, *Barrett* (1) found infectious mononucleosis antibodies in 5 out of 300 blood donors (1.7 per cent). The value found in the present study, 1 per cent, is of roughly similar magnitude.

Experimental attempts at transferring infectious mononucleosis from one person to another have failed (6). A few suggestive results cannot be taken as certainties (8, 12, 15). This, and the fact that the infection seldom spreads to room mates and that no cross-infections occur in hospitals, seem to indicate a low risk of contagion (6, 8). The risk of infection through blood-bank blood must therefore be considered extremely improbable.

## SUMMARY

The incidence of sheep red-cell agglutinins in 321 blood donors was investigated. Eighty two (26 per cent) showed agglutinins to the titre ≧ 1/10. These sera were studied by the differential absorption test. Only in 3 cases (0.9 per cent) were the agglutinins of infectious mononucleosis type. This suggests that the true incidence of the disease is considerably higher than the number of diagnosed cases. The possible risk of infection through blood-bank blood is discussed.

## SERIES AND METHOD

The series comprised 321 blood donors, 221 of these passed through the Red Cross Blood Transfusion Service in Turku and 100 in Kuopio. For all donors the haemoglobin was not less than 12 gr % and the Kahn reaction was negative. 131 of them were men and 190 women. Their mean age was 35.5 ± 0.6 years.

The Paul-Bunnell test (presumptive test) was performed as follows. Serum was inactivated by keeping it at 56° C for half an hour. A dilution series of the serum was made in the ratio 1 : 2, 1/2.5 to 1/2560, 0.5 ml per tube. A 1 per cent suspension was made of sheep red cells washed three times with physiological saline solution. 0.5 ml of this suspension was added to each tube and, to serve as a control, to a tube of physiological saline. The sheep cells used were more than one day and less than 7 days old, cells were thus replaced weekly and derived from different sheep. New red cells were always tested with a Paul-Bunnell positive control serum. The final serum dilution was 1/5 to 1/5120. The tubes were incubated in a water bath for 2 hours at 37° C (16), after which the result was read with the naked eye. The degree of agglutination was assessed as follows: +++ = firm disc, ++ = disc easily broken into large flakes, + = finely granular agglutination.

*Differential absorption test.* In the event of an agglutination titre ≧ 1/10, absorption was carried out both by guinea pig kidney emulsion and by beef cells, followed by agglutination in the usual way. A titre ≧ 1/10 after guinea pig kidney absorption which disappeared on beef cell absorption was considered significant and to indicate infectious mononucleosis. The absorption was performed by 20 per cent guinea pig kidney emulsion in saline and 20 per cent beef red cell suspension in saline prepared by the *Mackie & McCartney* method (14). 1.25 ml of guinea pig kidney emulsion was added to 0.25 ml of serum and 1.25 ml beef cell suspension to another 0.25 ml batch of serum. The tubes were left for 1 hour at room temperature, centrifuged, and agglutination was performed as usual. The final serum dilutions were 1/10 to 1/10240.

## RESULTS

TABLE 1

*Presumptive Test with the Serum of 321 Blood Donors*

| | Titre | | | | | | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | [illegible] | 10 | 1/20 | 1/40 | 1/80 | 160 | 1/320 | 640 | 1/1280 | 2560 | 5120 | |
| Number | 149 | 89 | 35 | 35 | 10 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 321 |

TABLE 2

*Differential Absorption Test of 83 Sera*

| Treated with guinea pig kidney | | Treated with beef cells | | Total |
|---|---|---|---|---|
| Not absorbed | Absorbed | Not absorbed | Absorbed | |
| 3 | 80 | 0 | 83 | 83 |

The results of the presumptive test are given in Table 1. In 149 cases (46.5 per cent) the titre was less than 1/5. In about ¾ of the cases (74 per cent) it was less than 1/10. Only in 3 cases (0.9 per cent) were the red cell agglutinins of the infectious mononucleosis type. In 2 cases the titre was 1/20 and in one 1/320. In one of these cases (titre 1:20) the subject had a couple of months earlier had angina resistant to penicillin therapy; no streptococci, however, were found on culture

FROM THE DEPARTMENT OF MEDICAL MICROBIOLOGY UNIVERSITY OF TURKU FINLAND (HEAD PROFESSOR EERO MUSTAKALLIO M D)

# ABSORPTION PATTERNS IN THE DIFFERENTIAL ABSORPTION TEST FOR INFECTIOUS MONONUCLEOSIS

By

SIMO VIRTANEN

Received 20 i 62

Infectious mononucleosis antibody can be distinguished from the other factors which agglutinate sheep red cells by absorbing the serum with guinea pig kidney emulsion and beef red cells The following absorption formula is generally accepted (Table 1) (7 11 12 and 16)

TABLE 1
*Agglutinins for Sheep Erythrocytes in Human Serum (7 11 12 16)*

| Type of serum | Agglutination of sheep erythrocytes | | |
|---|---|---|---|
| | Unabsorbed | Absorbed with guinea pig kidney | Absorbed with beef red-cells |
| Normal | — or + | — | unchanged |
| Infectious mononucleosis | + | + | — |
| Serum sickness | + | — | — |

The results of absorption however have not in all cases been compatible with the above formula According to *Leibowitz* (6) normal serum gives the indicated pattern in 95 98 per cent of cases ...

... unrecognized cases of serum disease or ...

... persons The absorption was incomplete in every third person Only very rarely did beef red cell ...

... incubation at room temperature

[1] Aided by a grant from the *Rockefeller Foundation*

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## RESULTS

The whole material comprised 419 sera with titres $\geq 1/10$ listed in Table 3. All these sera were absorbed both with guinea pig kidney and with beef red cells. The results of absorption are given in Table 4. A total of 75 sera containing infectious mononucleosis antibodies were found

TABLE 3

*Titre of Presumptive Test $\geq 1/10$*

| Sera | Number |
|---|---|
| Submitted for Paul Bunnell test | 206 |
| Healthy blood donors | 83 |
| Adults submitted for routine Wassermann test | 26 |
| Hospitalized children | 104 |
| Total | 419 |

in the material. All these sera behaved in typical fashion, i.e., the antibody was not absorbed with guinea pig kidney but disappeared on beef red cell absorption. The agglutinations of all of the other sera disappeared both with guinea pig kidney and with beef red-cell absorption, an absorption pattern supposedly typical of serum sickness.

TABLE 4

*Differential Absorption Test*

| Sera | Treated with guinea pig kidney | | Treated with beef red-cells | | Total number |
|---|---|---|---|---|---|
| | Not absorbed = antibody of infectious mononucleosis | Absorbed | Not absorbed | Absorbed | |
| Submitted for Paul Bunnell test | 68 | 138 | 0 | 206 | 206 |
| Healthy blood donors | 3 | 80 | 0 | 83 | 83 |
| Submitted for routine Wassermann test | 1 | 25 | 0 | 26 | 26 |
| Hospitalized children | 3 | 101 | 0 | 104 | 104 |
| Total | 75 | 344 | 0 | 419 | 419 |

Since it could be assumed that the atypical result of absorption with beef red cells might be attributable either to the red cell suspension employed or to factors connected with its preparation, ten sera with agglutinins which were not of the infectious mononucleosis type were absorbed with boiled red cells from ten cows (Table 5). Twelve sera were absorbed with crude red cells from four cows (Table 6). The agglutinins were completely [illegible]ed in all of the cases.

Barrett's series of 300 healthy blood donors included 10 in whom the sheep red cell agglutinins did not disappear with guinea-pig kidney or beef red cells (1). These cases, therefore, did not conform to the formula at all. Barrett employed a technique in which the tubes were centrifuged at a speed between 2,000 and 2,500 r.p.m. for 5–10 min., and left at room temperature for 15 min., after which the result was read.

For many patients who had had serum treatment *Dempster* (3) found that the antibody which agglutinated sheep red cells did not disappear completely through absorption with guinea-pig kidney or beef red cells. Dempster's series also included a case in which the patient was not affected with mononucleosis and had been given no serum treatment, yet the antibody resisted absorption with both antigens. Dempster's results were obtained after overnight refrigeration.

## MATERIAL AND METHOD

The material comprised 432 serum samples from patients with suspected infectious mononucleosis submitted in 1960–61 to the Department of Medical Microbiology, University of Turku, for a Paul Bunnell test; in addition there were 321 serum samples of healthy blood donors, 334 sera submitted for a routine Wasserman test and 207 serum samples from children treated at the Children's Clinic of Turku University. A number of the sera submitted for the Wasserman test derived from grown up patients at Turku University Central Hospital; the balance came from pregnant women attending the different Maternity Welfare Stations. None of the patients of the series had received serum treatment. Details of the patients are given in Table 2.

TABLE 2

*Material*

| Sera | Number |
|---|---|
| Submitted for Paul Bunnell test | 432 |
| Healthy blood donors | 321 |
| Adults submitted for routine Wassermann test | 334 |
| Hospitalized children | 207 |
| Total | 1294 |

Both the presumptive test and the differential absorption test were performed as previously described (18). The result was read after 2 hours of incubation in a water bath at 37° C. All titres are indicated in the final dilution of serum. In cases where the presumptive test titre was ≧ 1/10, absorption was performed with both guinea pig kidney and beef red cells.

Ten serum samples containing sheep red cell agglutinins which had disappeared on guinea pig kidney absorption were absorbed with boiled red cells from ten cows. The red cell suspension was prepared in the usual way. Four of the cows were Ayrshires (Ay breed) and 6 were of the West Finnish country breed (LSK breed); 4 belonged to blood group a and 6 to ab (8, 9 and 10). The blood group determination for the cows was performed by haemolytic reaction according to *Mustakallio & Rislakki* (8).

12 sera were absorbed with fresh red cells from 4 cows, of which 3 were Ayrshires and 1 of the West Finnish country breed. Two were of blood group a and the other two of ab.

TABLE 8

*Sheep Red Cell Agglutinins of 11 Sera before and after Absorption with Beef Red Cells*

| Titre before absorption at 37° C | | Titre after beef red-cell absorption | | | |
|---|---|---|---|---|---|
| | | at 37° C | at 4° C | | |
| | | < 1/10 | 1/10 | 1/20 | 1/40 |
| 1/10 | 7 | 7 | 1 | 1 | 5 |
| 1/20 | 3 | 3 | – | 1 | 2 |
| 1/40 | 1 | 1 | – | 1 | |
| Total | 11 | 11 | 1 | 3 | 7 |

20 sera, which in the presumptive test agglutinated to a relatively high titre at 20° C, were tested after absorption at 37° C, 20° C and 4° C. Absorption was performed as usual for 20° C. Guinea-pig kidney emulsion removed all the agglutinins, whereas beef red cells did not completely absorb the haemagglutinins active at 20° C and 4° C. The agglutination noted at 20° C and 4° C disappeared at 37° C.

## DISCUSSION

The absorption pattern of the "normal" sera tested differs from the formula usually given in text books (5, 7, 11, 12 and 16). Since the deviation could be assumed attributable to the beef red-cells employed, absorption was performed with boiled red cells from 10 cows of two breeds and two blood groups, and again with fresh red cells from 4 different cows. The result was constant: the agglutinins were always absorbed. It is obvious that absorption could not be exceptional, occurring only with certain beef red cells, but was the result of the antigen generally present in beef red-cells.

Why then had previous results suggested that the heterophil agglutinin in normal serum could not be absorbed with beef red cells? A possible explanation which would account for at least such results as

[illegible] in adults and some are still active at 20° C (17). Information on the temperature range of cold agglutinins is somewhat contradictory. According to *Kettel* (4) they are still active in some cases at 22° C but are no longer active at 27° C. According to *Sherman* (14) the critical point is 30–31.5° C, according to *Stratton* (15) 27–35° C. The temperature range of cold agglutinins widens with the height of their titre (13). Cold agglutinins with high titres are still active at 20° C but disappear at the latest at 32° C (13).

TABLE 5

*Absorption with Boiled Red-Cells from 10 Beeves*

| Number of sera | Titre before absorption | Absorbed by beef red cells | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ay breed | | | | I Sk breed | | | | | |
| | | ab | ab | ab | a | ab | ab | ab | ab | a | a |
| 1 | 1/20 | yes | yes | yes | yes | yes | yes | yes | yes | yes | yes |
| 4 | 1/40 | " | " | " | " | " | " | " | " | " | " |
| 3 | 1/80 | " | " | " | " | " | " | " | " | " | |
| 1 | 1/160 | " | " | " | " | " | " | " | " | " | " |
| 1 | 1/320 | " | " | " | " | " | " | " | " | " | " |
| 10 | | | | | | | | | | | |

Over 50 per cent of the sera with low sheep red-cell agglutinin titre at 37° C ($\leqq$ 1/5) agglutinated to a higher titre at 20° C (Table 7) This agglutination was reversible and disappeared after an hour of incubation at 37° C The factors involved were obviously cold agglutinins which were still active at 20° C

TABLE 6

*Absorption with Crude Red Cells from 4 Beeves*

| Number of sera | Titre before absorption | Absorbed by beef red cells | | | |
|---|---|---|---|---|---|
| | | Ay breed | | | I Sk breed |
| | | a | a | ab | ab |
| 6 | 1/10 | yes | yes | yes | yes |
| 3 | 1/20 | " | | | |
| 1 | 1/40 | " | | | |
| 2 | 1/80 | " | " | " | " |
| 12 | | | | | |

Eleven sera (not of the infectious mononucleosis type) were absorbed with beef red cells and subsequently tested at both 37° C and 4° C Absorption removed the agglutinins active at 37° C but not those active at 4° C (Table 8) Beef red cells, therefore, did not remove the cold agglutinins present in serum, but they were absorbed by guinea-pig kidney emulsion

TABLE 7

*Sheep Red Cell Agglutinins of 133 Sera at 37° C and 20° C*

| Titre at 37° C | Titre at 20° C | | | | | | | Total |
|---|---|---|---|---|---|---|---|---|
| | 1/5 | 1/5 | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | |
| < 1/5 | 42 | 22 | 40 | 13 | 3 | 1 | 0 | 121 |
| 1/5 | 0 | 0 | 2 | 6 | 3 | 0 | 1 | 12 |
| | 42 | 22 | 42 | 19 | 6 | 1 | 1 | 133 |

TABLE 8

*Sheep Red Cell Agglutinins of 11 Sera before and after Absorption with Beef Red-Cells*

| Titre before absorption at 37° C | | Titre after beef red-cell absorption | | | |
|---|---|---|---|---|---|
| | | at 37° C | at 4° C | | |
| | | < 1/10 | 1/10 | 1/20 | 1/40 |
| 1/10 | 7 | 7 | 1 | 1 | 5 |
| 1/20 | 3 | 3 | – | 1 | 2 |
| 1/40 | 1 | 1 | – | 1 | – |
| Total | 11 | 11 | 1 | 3 | 7 |

20 sera, which in the presumptive test agglutinated to a relatively high titre at 20° C, were tested after absorption at 37° C, 20° C and 4° C Absorption was performed as usual for 20° C Guinea-pig kidney emulsion removed all the agglutinins, whereas beef red cells did not completely absorb the haemagglutinins active at 20° C and 4° C The agglutination noted at 20° C and 4° C disappeared at 37° C

## DISCUSSION

The absorption pattern of the "normal" sera tested differs from the formula usually given in text-books (5, 7, 11, 12 and 16) Since the deviation could be assumed attributable to the beef red cells employed, *absorption* was performed with *boiled* red-cells from 10 cows of two breeds and two blood groups, and again with fresh red cells from 4 different cows The result was constant the agglutinins were always absorbed It is obvious that absorption could not be exceptional, occurring only with certain beef red-cells, but was the result *of the antigen* generally present in beef red cells

Why, then, had previous results suggested that the heterophil agglutinin in normal serum could not be absorbed with beef red-cells? A possible explanation, which would account for at least such results as

... range of cold agglutinins is somewhat contradictory According to *Kettel* (4), they are still active in some cases at 22° C but are no longer active at 27° C According to *Shermon* (14) the critical point is 30 31 5° C, according to *Stratton* (15), 27–35° C The temperature range of cold agglutinins widens with the height of their titre (13) Cold agglutinins with high titres are still active at 20° C but disappear at the latest at 32° C (13)

TABLE 9

*Sheep Red Cell Agglutinins of 20 Sera at 37° C 20° C and 4° C*

| Serum | Titre of presumptive test | | | Titre after absorption with guinea pig kidney | | | Titre after absorption with beef red cells | | |
|---|---|---|---|---|---|---|---|---|---|
| | 37° C | 20° C | 4° C | 37° C | 20° C | 4° C | 37° C | 20° C | 4° C |
| 1 | 1/5 | 1/20 | 1/40 | * | – | – | – | 1/10 | 1/20 |
| 2 | 1/5 | 1/20 | 1/20 | | – | – | – | 1/10 | 1/20 |
| 3 | 1/10 | 1/80 | 1/80 | – | – | | | 1/40 | 1/40 |
| 4 | 1/5 | 1/10 | 1/10 | – | – | – | | – | 1/10 |
| 5 | 1/5 | 1/20 | 1/20 | – | – | – | | 1/10 | 1/10 |
| 6 | 1/5 | 1/20 | 1/40 | – | – | – | | – | 1/20 |
| 7 | 1/5 | 1/20 | 1/20 | – | – | – | – | – | 1/10 |
| 8 | 1/5 | 1/40 | 1/160 | – | – | – | – | 1/20 | 1/80 |
| 9 | 1/5 | 1/40 | 1/40 | – | – | – | – | – | 1/20 |
| 10 | 1/5 | 1/80 | 1/80 | – | – | – | – | – | 1/10 |
| 11 | 1/10 | 1/20 | 1/20 | – | – | – | – | – | 1/20 |
| 12 | 1/10 | 1/40 | 1/40 | | – | – | – | – | 1/10 |
| 13 | 1/10 | 1/40 | 1/80 | – | – | – | – | – | 1/10 |
| 14 | 1/10 | 1/40 | 1/40 | – | – | | – | 1/20 | 1/20 |
| 15 | 1/20 | 1/20 | 1/80 | | – | – | – | 1/10 | 1/20 |
| 16 | 1/20 | 1/40 | 1/80 | | – | – | – | 1/20 | 1/40 |
| 17 | 1/20 | 1/80 | 1/80 | – | – | – | – | – | 1/20 |
| 18 | 1/20 | 1/40 | 1/160 | – | – | | – | 1/10 | 1/40 |
| 19 | 1/20 | 1/80 | 1/320 | – | – | – | – | 1/20 | 1/40 |
| 20 | 1/40 | 1/160 | 1/160 | | – | | | – | 1/20 |

* < 1/10

As is evident from Table 7, cold agglutinins still active at 20° C were found in about half the sera with a low titre at 37° C Cold agglutinins active at 4° C were not absorbed by beef red cells (Table 8) but were absorbed by guinea-pig kidney Table 9 shows that the absorption with *beef red cells of agglutinins active at 20° C and 4° C was incomplete,* while the agglutinins active at 37° C were totally absorbed by beef red cells (Tables 4, 5 and 9) It is evident, therefore, that the absorption pattern of a "normal" serum shown in Table 1 (no absorption with beef red cells or incomplete absorption (2)) is valid for determinations performed at refrigerator temperature or at room temperature, and depends on the cold agglutinins in the serum The anti-sheep red-cell agglutinins of "normal" serum, active at 37° C, are totally absorbed by beef red cells With the technique employed by the present writer, the

TABLE 10

*Agglutinins for Sheep Erythrocytes in Human Serum*

| Type of serum | Agglutination of sheep erythrocytes | | |
|---|---|---|---|
| | Unabsorbed | Absorbed with guinea pig kidney | Absorbed with beef red cells |
| Normal | — or + | – | — |
| Infectious mononucleosis | + | + | — |

action of cold agglutinins is eliminated by performing agglutination only at 37° C, and by reading the result immediately after agglutination. As regards the technique in which the result of the absorption is read after a 2-hour incubation at 37° C, an absorption pattern is shown at Table 10.

Since serum therapy is seldom applied to-day, the present material unfortunately contains no case of serum therapy. Therefore, the antibody absorption pattern in serum sickness cannot be commented on.

## SUMMARY

1,294 Paul Bunnell tests were performed after a 2-hour incubation at 37° C. A differential absorption test was performed in 419 cases in which the presumptive test had given a titre ≧ 1/10. A typical infectious mononucleosis antibody was found in 75 cases, in the remaining cases the antibody was absorbed both with guinea-pig kidney and with beef *red cells*. *An absorption* performed in the usual way with beef red cells removed from "normal" serum completely the sheep red-cell agglutinins active at 37° C, incompletely the cold agglutinins active at 20° C, but not those active at 4° C. The following absorption pattern is suggested for sheep red-cell agglutinin in "normal" serum: absorption both with guinea pig kidney and with beef red-cells.

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TABLE 9

*Sheep Red-Cell Agglutinins of 20 Sera at 37° C, 20° C and 4° C*

| Serum | Titre of presumptive test | | | Titre after absorption with guinea pig kidney | | | Titre after absorption with beef red-cells | | |
|---|---|---|---|---|---|---|---|---|---|
| | 37° C | 20° C | 4° C | 37° C | 20° C | 4° C | 37° C | 20° C | 4° C |
| 1 | 1/5 | 1/20 | 1/40 | –* | – | – | – | 1/10 | 1/20 |
| 2 | 1/5 | 1/20 | 1/20 | – | – | – | – | 1/10 | 1/20 |
| 3 | 1/10 | 1/80 | 1/80 | – | – | – | – | 1/40 | 1/40 |
| 4 | 1/5 | 1/10 | 1/10 | – | – | – | – | – | 1/10 |
| 5 | 1/5 | 1/20 | 1/20 | – | – | – | – | 1/10 | 1/10 |
| 6 | 1/5 | 1/20 | 1/40 | – | – | – | – | – | 1/20 |
| 7 | 1/5 | 1/20 | 1/20 | – | – | – | – | – | 1/10 |
| 8 | 1/5 | 1/40 | 1/160 | – | – | – | – | 1/20 | 1/80 |
| 9 | 1/5 | 1/40 | 1/40 | – | – | – | – | – | 1/20 |
| 10 | 1/5 | 1/80 | 1/80 | – | – | – | | – | 1/10 |
| 11 | 1/10 | 1/20 | 1/20 | – | – | – | – | – | 1/20 |
| 12 | 1/10 | 1/40 | 1/40 | | – | – | – | – | 1/10 |
| 13 | 1/10 | 1/40 | 1/80 | – | – | – | – | – | 1/10 |
| 14 | 1/10 | 1/40 | 1/40 | – | – | – | – | 1/20 | 1/20 |
| 15 | 1/20 | 1/20 | 1/80 | – | – | – | | 1/10 | 1/20 |
| 16 | 1/20 | 1/40 | 1/80 | – | – | – | – | 1/20 | 1/40 |
| 17 | 1/20 | 1/80 | 1/80 | – | – | – | – | – | 1/20 |
| 18 | 1/20 | 1/40 | 1/160 | – | – | – | – | 1/10 | 1/40 |
| 19 | 1/20 | 1/80 | 1/320 | – | – | – | – | 1/20 | 1/40 |
| 20 | 1/40 | 1/160 | 1/160 | – | | – | – | – | 1/20 |

* < 1/10

As is evident from Table 7, cold agglutinins still active at 20° C were found in about half the sera with a low titre at 37° C. Cold agglutinins active at 4° C were not absorbed by beef red cells (Table 8) but were absorbed by guinea-pig kidney. Table 9 shows that the absorption with beef red cells of agglutinins active at 20° C and 4° C was incomplete, while the agglutinins active at 37° C were totally absorbed by beef red cells (Tables 4, 5 and 9). It is evident, therefore, that the absorption pattern of a "normal" serum shown in Table 1 (no absorption with beef red cells or incomplete absorption (2)) is valid for determinations performed at refrigerator temperature or at room temperature, and depends on the cold agglutinins in the serum. The anti-sheep red-cell agglutinins of "normal" serum, active at 37° C, are totally absorbed by beef red cells. With the technique employed by the present writer, the

TABLE 10

*Agglutinins for Sheep Erythrocytes in Human Serum*

| Type of serum | Agglutination of sheep erythrocytes | | |
|---|---|---|---|
| | Unabsorbed | Absorbed with guinea pig kidney | Absorbed with beef red cells |
| Normal | — or + | — | |
| Infectious mononucleosis | + | + | |

MYCOLOGICAL SECTION DEPARTMENT OF BACTERIOLOGY, AND DEPARTMENT OF EPIDEMIOLOGY OF THE NATIONAL BACTERIOLOGICAL LABORATORY STOCKHOLM SWEDEN

# A CONTRIBUTION TO THE STUDY ON THE OCCURRENCE OF ADIASPIROMYCOSIS (HAPLOMYCOSIS) IN RODENTS IN SWEDEN

By

HEITI PALDROK and BO ZETTERBERG

Received 18 ii 62

*Adiaspiromycosis* is the name given by *Emmons & Jellison* in 1960 (7) to a new type of pulmonary mycotic disease, caused by *Emmonsia*, and so far found only in rodents and other smaller wild animals, such as mink, weasel, beaver, otter, etc

The term *Adiaspiromycosis* implies that there is no multiplication or dissemination of the fungus beyond its original site of implantation The fungal conidia simply grow in size in the invaded lungs, giving rise to thick-walled round sphaerules or adiaspores, the volume of which may become 1,000,000 times that of the conidium inhaled When present in very large numbers, the adiaspores may reduce effective lung tissue by mechanical displacement There is, on the whole, minimal host tissue response only during the early stages of growth However, chronic granulomatous lesions of the lungs caused by the sphaerules have been observed in an otter (17)

The disease was first observed by *Emmons & Ashburn* in 1942 (6) in rodents from Arizona, USA Taxonomically the authors assigned the fungus to *Haplosporangium* The causative agent was named *Haplosporangium parvum*, and for the lung condition the term *Haplomycosis* was proposed However, in 1959 *Ciferri & Montemartini* (3) changed the name of the fungus to *Emmonsia parva*, and as mentioned above, in 1960 *Emmons & Jellison* (7) proposed that the pathologic condition should be termed *Adiaspiromycosis*

*Emmons & Jellison* (7) assumed, that there are at least two species of *Emmonsia* which cause *Adiaspiromycosis*, *Emmonsia parva*, an uninucleate, and *E crescens*, a multinucleate, yielding adiaspores of 14 to 60μ versus 200 to 480μ, with a wall of 2μ versus 10 to 70μ

The type culture of *E crescens* originates from a rodent, a water vole, *Arvicola terrestris* captured at Hamar, Norway, in 1959 The two species appear also to be separated geographically According to *Emmons & Jellison* (7), *E parva* has been isolated only in the arid south west of the USA One collection, however, without culture, from Africa,

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Sweden, as listed in Table 1 The names of the animals in the table are given in accordance with the taxonomy of mammals by *van den Brink* (23)

TABLE 1

*Rodents Examined for Adiasporomycosis in Central Sweden during June October 1960*

| Rodents examined | Place of capture | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Uskaboda Västmanland county | | Hallboda Uppland county | | Burgsvik Gotland county | | Total | |
| | Number examined | Number infected | Number examined | Number infected | Number examined | Number infected | Number examined | Number infected |
| *Mus musculus* House mouse | 4 | – | 6 | – | 17 | – | 27 | – |
| *Sylvaemus (Apodemus) flavicollis* Yellow necked field mouse | 15 | – | 46 | 4 | | – | 61 | 4 |
| *Sylvaemus (Apodemus) sylvaticus* Longtailed field mouse | 10 | – | 20 | 1 | 4 | – | 34 | 1 |
| [illegible] | 6 | 1 | 4 | – | – | – | 10 | 1 |
| [illegible] | – | – | 5 | – | | – | 5 | – |
| [illegible] | 12 | – | 6 | – | – | – | 18 | – |
| [illegible] | 1 | – | – | | – | – | 1 | – |
| [illegible] | 1 | – | 25 | | – | – | 26 | – |
| | 49 | 1 | 112 | 5 | 21 | – | 182 | 6 |

The collection of animals was undertaken by one of us (*Zetterberg*) in connection with an investigation concerning the occurrence of RSSE (Russian Spring Summer Encephalitis) virus in animals in Sweden After preparing the part of the material for the virus investigation, the lungs of the trapped animals were preserved in 70 per cent alcohol, and sent to the laboratory for examination for the occurrence of fungi For the collection of the material and the identification of the animals we are indebted to *W Berg*, Conservator at the Museum of Natural History Stockholm, and Docent *B Lundholm*, Stockholm Further, we are indebted to *Y R Lundh*, Health Inspector, National Bacteriological Laboratory, for the technical arrangements

After an exposure for 24 hours in the laboratory to 2 per cent Na-hydroxide aqueous solution, the lungs were examined for the occurrence of adiaspores Attempts to cultivate the adiaspores found were not successful

As shown in Table 1, the infection was found in the lungs of 6 out of the 182 animals examined In 4 yellow necked field mice, *Sylvaemus (Apodemus) flavicollis*, one longtailed field mouse, *Sylvaemus (Apodemus) sylvaticus*, and one bank vole, *Clethrionomys glareolus* Each of the infected specimens contained one to six adiaspores varying from 250 to 395$\mu$ in size (Fig 1) Although no growth of the fungus had been

Kenya near Nairobi (13), may also be *E parva* (7). *E crescens*, on the other hand, has been reported not only from the northern part of the USA (8, 10) and Canada (1, 2, 4, 5) but also from Ecuador, South America (15) Further it has been encountered in Korea (11), and Japan (13) Nevertheless most of the observations on the occurrence of *E crescens* have been made in Europe: in England (19, 22), Sweden (12, 17), Norway (18), Finland (16), Germany (21), France (14) and Yugoslavia (15)

There is a report by *Caferri* (3) on the isolation of *Emmonsia* from soil in Italy The correctness of the identification of the fungus in an earlier report on its isolation from the soil in Missouri, USA, by *Menges & Habermann* (20), it doubted by *Emmons & Jellison* (7)

Concerning the occurrence of the fungus in Sweden, *Jellison* (12), in 1956 found *E crescens* in one of 31 wood mice, *Apodemus flavicollis*, collected near Gullgruva in Gavleborg county between 1954, and March 1955, and shipped to the USA for examination In April 1959, and partly February 1960, *Jellison, Vinson & Borg* (17) found five infected animals among 115 freshly trapped ones and 43 museum specimens in Sweden 2 wood mice, *Apodemus spp*, 1 vole, *Microtus*, 1 shrew, *Neomys fodiens*, and 1 otter, *Lutra lutra* One positive museum species had been collected as long ago as in 1845 Most of the animals came from the vicinity of Stockholm, situated in central Sweden, but a few were *from other areas lying in the most opposite directions from each other*, such as Luleå in the extreme north-, and Lund in the extreme south of the country The otter was found dead in the vicinity of Rattvik Kopparberg county, west-central Sweden, in February 1960

In another part of Scandinavia, *viz* in east-central Norway, in March 1959, during the examination of 562 fresh specimens of mammals, mainly rodents, *Jellison, Vinson & Holager* (18), observed the infection in 4 voles, *Microtus*, and one water vole, *Arvicola terrestris* In Finland, in April and May 1959, *Jellison, Helminen & Vinson* (16) found 10 infected animals among 49 fresh-, and 103 museum specimens one vole, *Microtus agrestis*, 2 muskrats, *Ondatra zibethica*, 7 water rats, *Arvicola terrestris*

*Emmonsia* has not been recognized as a human pathogen Nevertheless, because of the occurrence of *Adiaspiromycosis* in localities within epidemic areas of "*Nephropatia epidemica*" a disease of man of unknown etiology, *Jellison, Vinson & Holager* (18) suggested, that *Emmonsia* may be the etiological agent of "*Nephropatia epidemica*" in Scandinavian countries *However, no closer evidence in support of* this suggestion has yet been delivered

## OWN INVESTIGATIONS

The present investigation consists of an examination of the lungs of 182 smaller rodents, captured in the central and eastern part of central

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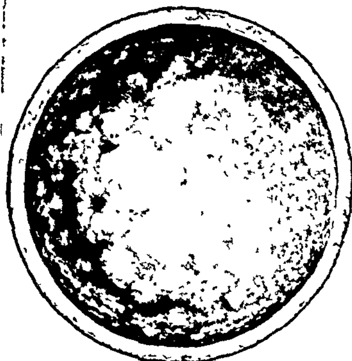


*Fig 1*
Adiaspore of 395 $\mu$ size

achieved, the adiaspores, on account of their size, were assumed to belong to the species *Emmonsia crescens*


SUMMARY

An examination of the lungs of 182 smaller rodents, captured during June to October 1960 in the central and eastern part of central Sweden revealed the presence of adiaspores in 6 of the specimens 4 yellow necked field mice, *Sylvaemus (Apodemus) flavicollis*, one longtailed field mouse, *Sylvaemus (Apodemus) sylvaticus* and one bank vole *Clethrionomys glareolus* Because of the size of the adiaspores encountered, 250 to 395$\mu$, the causative fungus was assumed to be *Emmonsia crescens*


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23 *Van*

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FROM KAPTEIN W. WILHELMSEN OG FRUES BAKTERIOLOGISKE INSTITUTT
OSLO UNIVERSITY AND RIKSHOSPITALET, OSLO

# PRODUCTION OF HYALURONIC ACID BY A STRAIN OF STREPTOCOCCUS ZYMOGENES

By

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Received 8.iii.62

Production of hyaluronic acid by streptococci belonging to *Lancefield's* groups A and C is well known, but we have been unable to find any record of production of this polysaccharide by strains belonging to the enterococci. Such a strain was recently isolated from a sample of urine.

The patient was a boy of 15 who had undergone a plastic operation on the left ureter for nephrolithiasis and hydronephrosis. After the operation he had for some time been treated for urinary infection with sulphonamides and nitrofurantoin. The sample of urine on direct microscopy was found to contain numerous granulocytes and grampositive oval cocci in short chains. Cultures on blood agar by the method of *Hoeprich* (3) yielded growth of more than 10⁵ colonies pr. ml of the strain described in the following.

## DESCRIPTION OF STRAIN

*Microscopy.* Round grampositive cocci in pairs and short chains. Stained by the method of *Butt et al.* (1) the organisms were found to be surrounded by capsular spaces of variable size, but mostly rather narrow.

*Culture.* Blood agar colonies (Figs. 1 and 2) were strongly mucoid, confluent and amoeboid, and surrounded by wide zones of β-haemolysis. The cultures were very like cultures of the mucoid form of Str. pyogenes. Growth was vigorous on all the usual media. Broth cultures were evenly turbid and contained short chains of nonmotile cocci.

*Biochemical reactions.* Acid was produced from glucose, mannitol, maltose, saccharose, salicin and trehalose, from sorbitol within 48 hours and from lactose after 5 days. Inulin and raffinose were not fermented, and gelatin was not liquefied. In glucose peptone water after 4 days at 30° C a questionable reaction for acetoin (faint pink color) was obtained by *Barritt's* method.

*Resistance.* The strain grew well on 40 per cent bile blood agar, in 6.5 per cent NaCl in broth and on *McLeod's* tellurite medium. It sur-

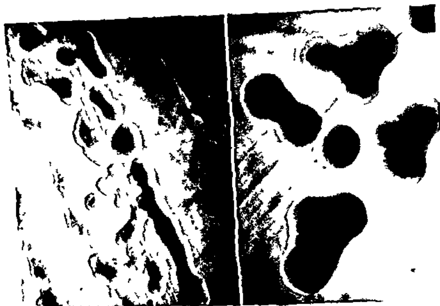


*Fig 1*
Blood agar culture, × 6

*Fig 2*
Blood agar culture × 10

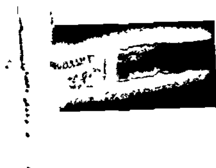


*Fig 3*
Effect of staphylococcal hyaluronidase The streptococcal hyaluronic acid is depolymerised next to the staphylococcus streak

vived heating to 56°C for 1 hour The terminal pH in glucose broth after 4 days was 3 9 It was resistant to penicillin Detection of hyaluronic acid When hyaluronidase-producing staphylococci and the strain were cross-streaked on blood agar, the growth was rendered non-mucoid for a distance of about 0 5-1 5 cm from the staphylococcus streak (Fig 3)

*Serology* Several attempts were made to determine the serological group of the strain, but it failed to react with the sera of groups A through O

*Haemolysin* In spite of the very strong haemolysis on blood agar a soluble haemolysin could not be detected in 18 h cultures in ordinary broth or in 20 per cent rabbit serum broth

*Classification* The strain behaved as Streptococcus zymogenes in all respects, except that is was mucoid and failed to react with group D immune serum

## PREPARATION AND ANALYSIS OF THE CAPSULAR POLYSACCHARIDE

Five l of a 24 h culture in broth containing 0 15 per cent glucose, to which 1 per cent phenol had been added, were centrifuged in the cold, filtered through a Berkefeld filter and concentrated to about 1/10 volume by vacuum distillation The polysaccharide was isolated and purified by the method of *Kendall, Heidelberger & Dawson* (4) The yield was 0 459 g The product was a pure white substance which gave clear, highly viscous solutions in water

Analytical data (figures from previous analyses of hyaluronic acid are shown in parantheses) N 4 26 per cent (3 3 8) P 0 48 per cent (O) Acetyl 9 4 per cent (9 5–11) $(\alpha)^{20}_{D}$ —70° (—47° to—78°)

A solution of the substance produced a precipitation of horse serum by the method described in (7)

A bovine testicular extract prepared as described in (5) caused a reduction of the viscosity of a solution nearly to that of the solvent

*Chromatography* A sample of the polysaccharide was hydrolysed with 3 N $H_2SO_4$ at 100° C for 6 h, neutralized with $BaCO_3$, and chromatography was carried out on the supernate The following solvent systems were used pyridin ethyl acetate water (2 5 5) and pyridin ethyl acetate acetic acid water (5 5 1 3) The chromatograms were stained with aniline oxalate and with ninhydrin A sample of commercial hyaluronic acid was used as a control

In the chromatogram which was stained with ninhydrin, the two preparations showed corresponding spots one distinct spot, apparently representing glucosamine, and several additional spots, one of which showed the same motility as galactosamine, whereas the others were less motile These additional spots may represent products of incomplete hydrolysis or of destruction during hydrolysis The main point seems to be the practically identical chromatograms of our polysaccharide and the standard

In the chromatograms stained with aniline oxalate both preparations showed a distinct spot corresponding to glucuronic acid, a second spot with the same location as a galacturonic acid standard, and a weak

glucose spot The streptococcus polysaccharide in addition showed a very strong spot corresponding to galactose in both solvent systems and a faint spot which appeared to be fucose Thus the streptococcus polysaccharide contained two monosaccharides in addition to those contained in the hyaluronic acid standard In view of the other results which were obtained the most likely explanation seems to be that the streptococcus product was a mixture of hyaluronic acid and another polysaccharide

An attempt was made to demonstrate the monosaccharide components of hyaluronic acid after enzymatic hydrolysis according to the method of *Meyer Linker & Rapport* (5) but although the enzyme preparation caused a marked reduction of viscosity we failed to demonstrate any break down products in a dialysate possibly because too small quantities of polysaccharide were left for the test

## DISCUSSION

The results indicate that the strain produces a hyaluronic acid capsule The chromatograms suggest that the polysaccharide isolated from a broth culture may have been contaminated with a second polysaccharide containing galactose and fucose neither of which sugars is present in hyaluronic acid

In their paper on the dissociation of haemolytic streptococci *Dawson Hobby & Olmstead* (2) describe an M form of Str faecalis The description and the photographs of this M form suggest that it differs from our strain Apparently it was not nearly as strongly mucoid as our strain nor was any evidence presented that the capsule consisted of hyaluronic acid It seems that the M form described by *Dawson et al* corresponds to the usual colony type of Str faecalis

In his extensive studies on enterococci *Skadhauge* (8 9) found that some strains of Str faecium had small capsules consisting of serologically active type specific capsular antigens In Str faecalis he demonstrated a special surface antigen designated the K antigen but he did not encounter mucoid nor encapsulated strains

*Nyman* (6) searched for encapsulated strains of enterococci but did not find any He reexamined one of *Dawson's* strains without being able to demonstrate capsules

We have failed to find any reference to the isolation of enterococci producing hyaluronic acid in the literature of recent years

Although our strain did not give any reaction with the grouping sera available (sera of commercial origin) we feel th

*Serology* Several attempts were made to determine the serological group of the strain, but it failed to react with the sera of groups A through O

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# IN VITRO PRODUCTION OF ANTIBODIES BY THORACIC DUCT LYMPHOCYTES

By

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Received 16 iii 69

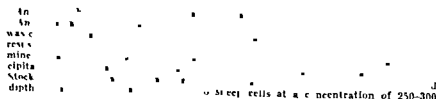
It has been clear for a long time that the lymphatic cell system is of great importance in antibody production

When comparing the efferent and afferent vessels of the lymph nodes draining an antigen stimulated area *Ehrich & Harris* (1942) found that the efferent vessels contained a higher antibody titre As has been demonstrated repeatedly cells from lymph nodes and spleens of immunized animals produce antibodies when grown in tissue cultures (*Fagraeus* 1948 *Thorbecke & Keuning* 1953 *Stavitsky* 1955 and several others) or when transferred into non immunized animals (*Harris Harris & Farber* 1954 *Harris & Harris* 1954 *Weigle & Dixon* 1959 *Nossal* 1960 and several others)

Most authors now agree that the cells involved in antibody production are of several types namely plasma cells (*Fagraeus* 1948) lymphocytes (*Wesslen* 1952 a) and peritoneal exudate cells (*Stevens & McKenna* 1960)

However a study of this problem shows that only a few investigators (*Wesslen* 1952 a b *Holub* 1958 1960) have used the relatively uniform lymphocyte suspension which can be obtained from the thoracic duct In the work presented here the ability of these cells to produce antibodies in vitro has been closely studied and antibodies have been demonstrated by means of different immunological techniques (agglutination haemagglutination bacterial adhesion and fluorescent antibody techniques)

## MATERIALS AND METHODS



SUMMARY

A mucoid strain of Streptococcus zymogenes, producing hyaluronic acid, was isolated from the urine of a patient suffering from infection of the urinary tract.


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8 per cent) After centrifuging and washing the sediment was resuspended to a volume of 37 ml with saline containing ½ per cent normal rabbit serum (*Scheibel* 1960)

*Production of Antisera and Conjugation with Fluorescent Substance:*

a) Rabbit antisera specific to Esch coli O127 B8 and O128 B 12 were obtained by the procedure laid down by *Edwards & Ewing* (1957) The O agglutination titre was about 1/2560 and the B agglutinin titre 1/124 The globulin fraction was precipitated

orange fluorescence

b) Diphtheria antitoxin was obtained by injecting rabbits with alum precipitated diphtheria toxoid as follows A total dose of 150 Lf was given once a month during a period of 3 months Of this dose 130 Lf was administered in several doses intra-

...ocyanate as laid down by *Riggs et al* (1958) The unconjugated substances were removed by passing the sera through a column packed with Sephadex G-25 (Pharmacia) and finally the conjugates were adsorbed twice with mouse liver-powder according to *Coons* (1958)

*Preparation and Staining of Slides*

a) Staining of antigen The smears were air dried and fixed with methanol for 15 minutes One drop of conjugated gammaglobulin specific to the bacteria used was placed on each smear which was left in a moist chamber at room tempe...

...ashed once more and mounted as above

*Fluorescence M...*

## RESULTS

1 *Antibodies against Cellular Antigen:* When the lymphocytes obtained from rabbits immunized with Esch coli 0127 B 8 and operated on the second day after the last injection had been incubated a titre of up to 1 16 was obtained in the culture medium, see Fig 4 Control of the last washing fluid gave no titre at all

Lf/ml equivalent to 0 6–0 9 mg protein/ml was kindly placed at our disposal by Dr Scheibel of the Danish State Serum Institute Copenhagen

*Medium* A complex amino acid-vitamin salt mixture having no antigenic properties was used (Parker 199 – American Public Health Association 1956)

*Immunization of Rabbits before Collection of Cells from the Thoracic Duct*
1) Immunization with Esch coli O127 B8 and O128 B12 was carried out according to the procedure laid down by *Edwards & Ewing* (1957) (2) Immunization with the alum precipitated diphtheria toxoid was produced in the following way 85 Lf was given in multiple injections intramuscularly in the hind legs and simultaneously 15 Lf was injected intravenously In animals to be studied for secondary response the procedure was repeated after 4 weeks (*Stavitsky* 1954)

*Collection of Lymph* (*Wessten* 1952 b)

The rabbits were devided into two groups Those belonging to the first group were investigated on the 3rd to 10th days during primary response, those in the second on the 1st to 8th days during secondary response

The animals were anaesthetized with nembutal (Abbot) the hair of the site of operation was removed by barium sulphide, and the skin locally anaesthetised with xylocain 0 5 per cent + norexadrin (Astra) An incision was made above the junction of the subclavian and jugular veins on the left side, the muscles removed by diathermy, and the thoracic duct freed and punctured The lymph was now drawn into a heparinized syringe Thereupon the animals were killed

*Preparation of Lymphocyte Suspension*

The lymph so collected was centrifuged at 1 500 r p m and the sediment washed 3 times in Parker's solution The lymph and supernatant fluids from the washings were withheld for antibody determinations The cells were counted and the number of viable cells estimated by a viability test as described below All lymph specimens were tested for sterility and samples showing bacterial growth were discarded

*Viability Test* (*Schrek*) 0 2 ml suspension of lymphocytes was mixed with 3 8 ml of Tyrode's solution to which eosin had been added at a concentration of 1/1000 The number of unstained cells was determined in a Burker counting chamber

*Cell Culture* (1) The suspensions of washed lymphocytes obtained from rabbits immunized intravenously with Esch coli O127 B8 were resuspended in Parker's solution and yielded a count of 40 000 60 000 cells/mm$^3$ medium The suspension was devided into three parts The first was cultured in roller tubes (2 ml per tube) at 37° C and subsequently the O agglutination titres were determined in certain cases after 9 in others after 17 hours incubation

Another part of the lymphocyte suspension was mixed with Esch coli O127 B8 As a control a similar portion was incubated with Esch coli O128 B12 which is antigenically different from Esch coli O127 B8 A bacterial density of 15 20 bact/lymphocyte was used The mixtures were incubated at 37° C in roller tubes or L glasses as modified by *Reiss Mertens & Ehrich* (1950) Smears were made after 60 and 100 minutes and stained with fluorescent antibodies as described below The number of organisms adhering to the lymphocytes was easily observed with the fluorescence microscope

(2) Washed lymphocytes from the thoracic duct of rabbits immunized with diphtheria toxoid were incubated in small tubes—one for each sample in the absence of antigen The samples were subjected to the following examinations pH estimation counting of cell number and estimation of the number of viable cells by the viability test

*Antibody Titrations*

a) The Esch coli O127 B8 antisera titrations were made using the agglutination techn [illegible] (1957)

b) [illegible]

techn [illegible]

used to adsorb [illegible] diphtheria toxoid of the type previously described was mixed with 26 ml of phosphate buffer, pH 6 4 and 9 2 ml suspension of tannic acid treated sheep cells (about

When washed thoracic duct cells from rabbits immunized with Esch coli 0127 B 8 were incubated with Esch coli 0127 B 8 bacterial adhesion was found to occur around a majority of the cells, as seen in Fig 1 Esch coli 0128 B 12 were used as control bacteria and gave quite a different picture (Fig 2) A quantitative examination was made and the results shown in the table in Fig 3 Here the cells have been divided into groups according to the number of bacteria adhering to each lymphocyte The number of cells in each group is expressed as a percentage of 500 cells counted It is evident from the table that Esch coli *0127 B 8 has a strong tendency to adhere to thoracic duct lymphocytes* from the homologously hyperimmunized rabbits A certain unspecificity in this phenomenon, however, can be noted, as, to a certain extent Esch coli 0128 B 12, also adhered to the lymphocytes of the rabbits immunized with Esch coli 0127 B 8

| Bacteria per cell | Homologous strain O 127 B8 | | Heterologous strain O 128 B12 | |
|---|---|---|---|---|
| | 60 min | 100 min | 60 min | 100 min |
| 0 5 | 17 2 | 33 2 | 11 0 | 11 2 |
| 6 10 | 17 | 15 6 | 0 | 0 |
| 11 15 | 12 6 | 5 7 | 0 | 0 |
| 16 20 | 3.2 | 0 | 0 | 0 |

*Fig 3*

A quantitative examination of the bacterial a[illegible]
cubation time
incubated with
divided into gro
The number of

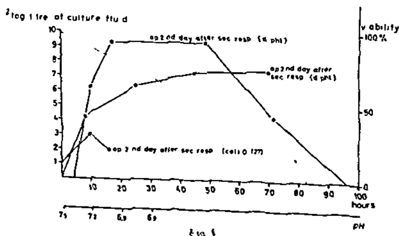



*Fig 4*

Antibody titres of the culture media after different periods of incubation in relation to cell viability and pH After 30 hours the pH is 6 9

● ● Antibody titre Viability of cells



*Fig 1*

Washed thoracic duct cells from rabbits immunized with Esch coli 0127 B 8 incubated with Esch coli 0127 B 8 The bacteria have a strong tendency to adhere to the lymphocytes



*Fig 2*

Control Washed thoracic duct cells from rabbits immunized with Esch coli 0127 B 8 incubated with Esch coli 0128 B 12 The cells are free from bacteria

secondary response, while no antibody titres were obtained after operations on the 3rd, 5th, 7th and 10th days during primary response The experiments during secondary response were repeated from days 1-5 and the same results were obtained

In this second series we were also interested in the occurrence of antibodies within the cells After incubation the cells were centrifuged and the supernatant fluid decanted The cells were then washed and lysed and finally centrifuged at 3 000 r p m for 30 minutes to remove the cell debris

| Number of days after (last) inj | Antibodyformation | |
|---|---|---|
| | Prim resp | Sec resp |
| 1 | | — |
| 2 | | + |
| 3 | | + |
| 4 | | + |
| 5 | — | — |
| 6 | | — |
| 7 | — | — |
| 8 | | — |
| 9 | | |
| 10 | — | |

*Fig 6*

Animals operated on various days during primary and secondary response A plus sign shows that thoracic duct cells from the animals in question were able to form antibodies *in vitro*

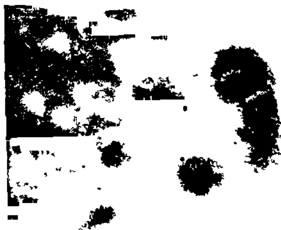


*Fig 7*

Thoracic duct cells from rabbits immunized with diphtheria toxoid and treated with isothiocyanate labelled *diphtheria antitoxin* (Sandwich technique) Positive cell at the top right corner of the picture

2 *Antibodies against Soluble Antigen* Wesslen (1952 a) showed that thoracic duct lymphocytes form antibodies during secondary response In order to find the optimum conditions for this antibody formation we investigated some of the animals immunized with diphtheria toxoid on the second and third days of secondary response The washed thoracic duct lymphocytes were incubated in small tubes as described earlier and the antibody-content of the centrifuged culture medium was then estimated The results shown in Fig 4 are from rabbits investigated on the second day after the second injection

There was a rise in titre during the first 24 hours, in a few cases up to 1/640 Thereafter the titre remained stationary or occasionally disappeared completely for some unknown reason About the same time the titres stopped rising the viability test showed 50–85 per cent viable cells compared to about 95 per cent viable cells at the start of the experiment The pH dropped from 7 5 to 6 9 In our attempts to adjust the pH we failed to keep the titre rising

Control experiments with lymphocytes from non-immunized animals were made simultaneously and no titres were obtained The washings gave no titres either

| Rabbit op on third day sec resp | | | |
|---|---|---|---|
| Incubationtime hours | 20000 cells | 60000 cells | 120000 cells |
| 0 | 0 | 0 | 0 |
| 5 | 0 | 10 | 20 |
| 17 | 40 | 160 | 20 |
| 42 | 80 | 320 | 20 |
| 65 | 40 | 320 | 20 |

*Fig 5*

Antibody titres of the culture media in relation to the number of incubated cells

Fig 5 shows how the titres vary with the number of cells incubated With a high concentration of cells antibody titres were obtained as early as after 5 hours incubation, upon which they remained stationary whereas lower cell concentrations developed antibody titres at a slower rate but they retained their antibody-producing capacity longer The optimum number of cells seems to be about 60 000 cells/mm³, which is approximately the cell concentration in the thoracic duct

The results obtained in the earlier experiments were utilized for another series aimed at investigating the time limits for antibody formation Some animals were operated on various days during primary response, others on various days during secondary response The results are shown in Fig 6 Lymphocytic antibody production was demonstrated on the 2nd, 3rd and 4th after the last injection during

timum number of cells seems to be 60 000 cells/mm³ the same concentration as found in the thoracic duct which also agrees with Wesslen's results

During secondary response antibody production could be demonstrated only on the 2nd 3rd and 4th days while no antibody production at all was demonstrated during primary response This differs from the results of in vitro experiments with spleen cells and lymph node cells *Fagraeus* (1948) for example could demonstrate maximum antibody production on the 4th 5th day after re injection and could still demonstrate antibody production in samples taken on the 7th day She also established antibody production in samples taken during primary response after waiting for at least 3 days after the injection *McKenna & Stevens* (1960) demonstrated antibody production against bovine gammaglobulin by peritoneal exudate cells during both primary and secondary response In both cases the cells were collected on the 4th day after antigen injection Using the fluorescent antibody technique *White* (1960) could show that during primary response antibodies occurred only in plasma cells in the medulla of the regional lymph node In lymphocytes in the cortex antibodies were demonstrated only after a second dose of antigen had been given Our series of experiments had its limitations Another way of tackling the problems would be to insert a draining tube into the thoracic duct of an animal and follow through the immunization period on the same animal

The fact that bacteria have a tendency to aggregate around homologously — but in connect 1950 *Hayes* n was thought to have some connection with antibody production as the bacteria aggregated around plasma cells Besides the specific agglutination around lymphocytes Esch coli of the type used in our experiments has a tendency to aggregate unspecifically around the lymphocytes

In his experiments *Wesslen* could not demonstrate antibodies from washed lysed lymphocytes which had the ability to produce antibodies In rabbits hyperimmunized with ovalbumin *Fuji* (1958) could extract antibodies against ovalbumin from plasma cells from the omentum but not from thoracic duct cells treated in the same way The method used by us made it difficult to estimate the antibody content within the cells owing to an unspecific haemagglutinating factor The titration of supernatant culture medium never gave any such unspecific haemagglutination

Using the fluorescent antibody technique we observed antibodies within some round cells in smears from suspensions of cultivated thoracic duct cells The same technique made it possible for other authors to observe antibodies in plasma cells in spleen and lymph nodes (e g *Coons et al* ) and in thoracic duct cells incubated in a diffusion chamber in the peritoneum of a rabbit (*Holub* 1960) On the

The cells were lysed partly by means of the ultrasonic disintegrator and partly using distilled water However, this procedure, probably released an unspecifically haemagglutinating factor from the cells, as controls with lymphocytes from nonimmunized animals gave positive titres These titres occurred regularly after lysis with the ultrasonic disintegrator, but not so often after lysis with distilled water Supernatant fluids of lymphocytes cultures from nonimmunized animals gave no titres

For the follow-up cell smears were made from all of the experiments and stained by means of the fluorescent antibody technique It was our intension to be able to demonstrate possible intracellular antibodies with this method Unfortunately we could not prevent a certain degree of unspecific fluorescence in the cells A few cells differed from the others by having an intensely fluorescent cytoplasm These cells were relatively few, as seen in Fig 7 (See further under discussion)

## DISCUSSION

As mentioned earlier most authors agree that more than one type of cell is involved in antibody production However, the intrinsic significance of the morphologically different types of cells is not clear *Fagraeus* (1948) showed, that spleen cells from immunized animals could produce antibodies in cell culture She was succesful also in correlating a rise in antibody titre with the appearance of plasma cells *Wesslen* (1952 a) showed that thoracic duct lymphocytes from an animal immunized with S typhi H antigen produced antibodies against this antigen in cell culture Since no antibodies could be demonstrated in the lysed cells he found that the lymphocytes could produce antibodies without themselves containing any appreciable amount *Holub* (1960) in his experiments incubated normal lymphocytes together with an antigen in a diffusion chamber intraperitoneally in 5 day old rabbits, which themselves cannot produce antibodies, and demonstrated antibody production

*McKenna & Stevens* (1960) cultured peritoneal exudate cells (mainly monocytes) from rabbits immunized with one or more injections of bovine gammaglobulin and found that these cells too had antibody-producing capacity

In the present study using different immunological techniques, we have been able to confirm Wesslen's results viz that under certain circumstances thoracic duct lymphocytes produce antibodies in vitro

Under our experimental conditions the cells rapidly lost their vitality and no further rise in titre occurred after a few days' incubation This could be due to the accumulation of acid waste products which presumably caused the drop in pH Early termination of antibody production also occurred when a large number of cells were incubated, which means a greater accumulation of acid waste products The op

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basis of his results *Holub* considered that lymphocytes could change into antibody producing plasma cells. This opinion is shared by others (*Neil & Dixon* 1959, *Fichtelius* 1960) and thus it would perhaps be wise not to differentiate too sharply between lymphocytes and plasma cells.

A cautious interpretation of our data does not conclusively establish that the antibodies are produced by the lymphocytes because of the difficulties to estimate antibody content of lysed cells. With regard to the significant difference of antibody content of lymphocyte cultures from secondary and primary response and from cultures of different days during secondary response it appears likely, however, that the antibodies are produced by the cells. Thus the present study gives some evidence that the thoracic duct provides the blood with antibody producing cells.

It was our intention from the beginning to try to estimate how many of the lymphocytes entering the blood stream under certain conditions are antibody producers. A number of factors complicated this task. In order to utilize the ability of the bacteria to aggregate round homologously immunized lymphocytes the cells had to be incubated together with the antigen, which increased aggregation and thus made the results difficult to read. Aggregation may also account for the lower percentage of positive cells obtained after a long period of incubation. When smears are made it is difficult to get the aggregations to spread evenly. Moreover the adhesion phenomenon does not seem to be quite specific. These difficulties do not arise with the use of soluble antigens, but here experiments made by *Boyden & Sorkin* (1960) have shown that non antibody producing spleen cells can be "infected" by the extract from antibody producing cells and then specifically adsorb the antigen. This makes the fluorescent antibody technique unsuitable for a quantitative estimation of the number of antibody producing cells in the thoracic duct. A more suitable technique in this connection would probably be the single cell method devised by *Nossal* (1958).

## SUMMARY

The capacity of thoracic duct lymphocytes to produce antibodies has been studied in vitro. The conventional agglutination and haemagglutination techniques were used to determine the optimum conditions for antibody formation by these cells. Thus the production of antibodies could be noted on the 2nd, 3rd and 4th days during secondary response. Methods for a quantitative determination of the number of antibody producing thoracic duct lymphocytes in a given situation were tried out (bacterial adhesion technique, fluorescing antibody technique). The relation of these lymphocytes to other antibody producing cells is discussed.

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# TYPE 48, A NEW PNEUMOCOCCUS TYPE

*By*

ERNA LUND

Received 24 iii 62

The latest publication on serological classification of pneumococci (2) gives the number of types as 80. In 1960 *Kauffmann, Lund & Eddy* (1) presented a correlation between the Danish and the American nomenclature for the 80 types known at that time. The following describes a new Pneumococcus type, which has been included in the Danish nomenclature as Type 48. Up to now this type has been isolated only once in our laboratory and has not been described by others.

The new type was isolated as a pure culture from a pleural exudate of a male patient aged 48 years. The strain (No 378/60) is morphologically a typical Pneumococcus, it is sensitive to optochin, and ferments glucose, galactose, lactose, sucrose, maltose, inulin, salicin and esculin within 24 hours, but does not ferment arabinose, xylose, dulcitol, inositol, sorbitol, mannitol or adonitol after five days. Type 48 is slightly virulent in the mouse test.

With nine pooled diagnostic sera (3), including all 80 types, the new strain gave no capsular reaction (Neufeld reaction). A serum broth culture of the strain was examined with unabsorbed antisera of the 80 recognized Pneumococcus types and gave only a weak capsular reaction with Type 24 A. This reaction was negative when the serum was diluted 1:2. The strain was not agglutinated by undiluted serum 24 A. A formol vaccine (3) of the strain was examined in the same way, and here weak reactions were found with three types: 15 A, 19 C and 24 A. The titers are given in Table 1.

TABLE 1

*Cross Reactions with Strain and Serum of Type 48*

| Type 48 | Cross-reactions with types | |
|---|---|---|
| | Capsular reactions | Agglutination reactions |
| Strain (Vaccine) | 15A (4) 19C (4) 24A (16) | 15A (2) 19C (4) 24A (16) |
| Serum (1 month) | no reactions | no reactions |
| Serum (8 months) | 19C (8) | 19C (8) |

The figures in brackets give the titers

A Pneumococcus group 24 rabbit serum (prepared by immunization for seven months with types 24, 24 A and 24 B) showed a capsular titer of 32 and an agglutination titer of 16 with vaccine of Type 48

An anti pneumococcal serum was prepared with the new strain (3) After immunization of 10 rabbits for 1 month, the serum showed a homologous capsular titer of 512 and an agglutination titer of 2048 This serum was examined for capsular reactions with vaccines of all 80 Pneumococcus types with negative results Even with vaccine prepared of Type 24 A there was neither capsular nor agglutination reaction After immunisation for 8 months 3 of the animals remained and were bled Their serum had a homologous capsular titer of 2048 and an agglutination titer of 4096, and showed only one heterologous reaction, namely with Type 19 C (capsular and agglutination titer 8 (Table 1))

Types 19 C and 48 thus may possess a common antigen, but as the cross reactions for both antigen and antiserum are weak, this antigen will not be included in the formula for Type 48 The reactions with Types 15 A and 24 A are only seen with the antigen of Type 48 and not with the antiserum and therefore these reactions will not be taken into account either The new type therefore has been given the following antigenic formula

*Type 48 = 48 a*

A new set of diagnostic Pneumococcus sera have been produced at Statens Seruminstitut These sera include 9 pooled sera (A–I) and 46 type or group sera numbers 1 to 48 (26 and 30 are not used) Type 48 is included in pool I and as a type serum 48

SUMMARY

A new Pneumococcus type has been established as Type 48 and included in the Danish nomenclature The type seems to be rare Its antigenic formular is 48 a

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# ON THE SPECIFICITY OF COMPLEMENT FIXATION TESTS FOR TYPING OF COXSACKIE VIRUS STRAINS

*By*

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Received 9.iii.62

In recent years increasing numbers of Coxsackie viruses have been reported as etiologic agents of human diseases, several of which present a severe clinical picture (1). It would therefore seem highly desirable to obtain a simple, quick, and economical method for the typing of virus strains freshly isolated from patients, and for this purpose the complement fixation (CF) test would seem to have certain advantages.

Theoretically the method might be employed in two ways (2). Either directly by using material containing the unknown virus as antigen in a CF test against known, specific antisera. Or indirectly, by immunizing animals with the unknown agent and subsequently examine the antiserum against known viral antigens. Since the preparation of antisera requires one or more weeks, it was assumed that the indirect procedure would be too time-consuming to be practical for routine diagnosis, which assumption was confirmed by pilot experiments in this laboratory (3).

Consequently, a series of experiments was commenced in order to work out a simple method which would make suspensions of infected tissue from newborn mice suitable for use as antigens in CF tests. In a preliminary report (4) experimental preparation of such antigens has been described. Coxsackie viruses A1 and B1 recovered from stool specimens were chosen as representative strains for these studies. While the A1 strains were found to yield good CF antigens, results with the Coxsackie B1 strains were less satisfactory.

The present paper describes the results obtained with the adopted technique applied on all our prototype Coxsackie virus strains, i.e. Coxsackie A1 through 24 and Coxsackie B1 through 5. The Coxsackie strain B6 was not available in our laboratory at the time of the experiments.

The help and advice by *Dr. Herdis von Magnus* in the preparation of the manuscript is gratefully appreciated, and so are suggestions to emendations in it made by mag. scient. *Halldor Thormar*.

The valuable technical assistance by *Miss F. Nahr, Mr. G. Mathiasen and Mr. H. Sørensen* is gratefully aknowledged.

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An anti-pneumococcal serum was prepared with the new strain (3) After immunization of 10 rabbits for 1 month, the serum showed a homologous capsular titer of 512 and an agglutination titer of 2048 This serum was examined *for capsular reactions with vaccines* of all 80 Pneumococcus types with negative results Even with vaccine prepared *of Type 24 A there was neither capsular nor agglutination re*action After immunisation for 8 months 3 of the animals remained and *were bled* Their serum had a *homologous* capsular *titer* of 2048 and an agglutination titer of 4096, and showed only one heterologous reaction, namely with Type 19 C (capsular and agglutination titer 8 (Table 1))

Types 19 C and 48 thus may possess a common antigen, but as the cross-reactions for both antigen and antiserum are weak, this antigen will not be included in the formula for Type 48 The reactions with Types 15 A and 24 A are only seen with the antigen of Type 48 and not with the antiserum and therefore these reactions will not be taken into account either The new type therefore has been given the following antigenic formula

*Type 48 = 48 a*

A new set of diagnostic Pneumococcus sera have been produced at Statens Seruminstitut These sera include 9 pooled sera (A I) and 46 *type or group sera* numbers 1 to 48 (26 and 30 are not used) Type 48 is included in pool I and as a type serum 48

SUMMARY

A new Pneumococcus type has been established as Type 48 and in*cluded in the Danish nomenclature The type seems to be rare* Its anti*genic formula is 48 a*

REFERENCES

1 *Kauffmann F Lund Erna & Eddy Bernice B* Proposal for a change in the nomenclature of Diplococcus pneumoniae and a comparison of the Danish and American type designation Internat *Bull* of *Bacteriol* Nomenclature and Taxonomy *10* 31 1960

2 *Lund Erna* The present status of the pneumococci including three new Pneumococcus types *Acta* path et microbiol scandinav *40* 425 1957

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# ON THE SPECIFICITY OF COMPLEMENT FIXATION TESTS FOR TYPING OF COXSACKIE VIRUS STRAINS

*By*

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In recent years increasing numbers of Coxsackie viruses have been reported as etiologic agents of human diseases, several of which present a severe clinical picture (1). It would therefore seem highly desirable to obtain a simple, quick, and economical method for the typing of virus strains freshly isolated from patients, and for this purpose the complement fixation (CF) test would seem to have certain advantages.

Theoretically the method might be employed in two ways (2). Either directly by using material containing the unknown virus as antigen in a CF test against known, specific antisera. Or indirectly, by immunizing animals with the unknown agent and subsequently examine the antiserum against known viral antigens. Since the preparation of antisera requires one or more weeks, it was assumed that the indirect procedure would be too time-consuming to be practical for routine diagnosis, which assumption was confirmed by pilot experiments in this laboratory (3).

Consequently, a series of experiments was commenced in order to work out a simple method which would make suspensions of infected tissue from newborn mice suitable for use as antigens in CF tests. In a preliminary report (4) experimental preparation of such antigens has been described. Coxsackie viruses A1 and B1 recovered from stool specimens were chosen as representative strains for these studies. While the A1 strains were found to yield good CF antigens, results with the Coxsackie B1 strains were less satisfactory.

The present paper describes the results obtained with the adopted technique applied on all our prototype Coxsackie virus strains, i.e. Coxsackie A1 through 24 and Coxsackie B1 through 5. The Coxsackie strain B6 was not available in our laboratory at the time of the experiments.

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The valuable technical assistance by *Miss F. Nohr, Mr. G. Mathiasen and Mr. H. Sorensen* is gratefully aknowledged.

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Types 19 C and 48 thus may possess a common antigen, but as the cross-reactions for both antigen and antiserum are weak, this antigen will not be included in the formula for Type 48 The reactions with Types 15 A and 24 A are only seen with the antigen of Type 48 and not with the antiserum and therefore these reactions will not be taken into account either The new type therefore has been given the following antigenic formula

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SUMMARY

A new Pneumococcus type has been established as Type 48 and included in the Danish nomenclature The type seems to be rare Its antigenic formular is 48 a

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serum dilution one litter of mice was employed each mouse receiving 0 02 ml intraperitoneally A simultaneous titration of virus was carried out The mice were observed daily for seven days and the titer was calculated as the highest initial serum dilution giving 100 per cent protection of the inoculated mice

*Preparation of CF antigen* For the preparation of a batch of antigen five litters of suckling mice were inoculated each mouse receiving an inoculum of 0 02 ml of virus suspension intraperitoneally The virus suspension was used in a dilution estimated to contain $10^{3}$—$10^{4}$ $ID_{50}$ pr inoculum an amount which we have found to produce typical signs of disease in the majority of the mice on the second or third day following inoculation At this time all of the animals were killed by etherization and stored at —20° C until used for preparation of CF antigen

The further procedure has been described in a previous paper (4) and only a brief review shall be given here After thawing the torsos of the mice were harvested a 20 per cent suspension was prepared and centrifugated to remove coarse particles In th present st d th [illegible]

slightly opalescent and yellowish—containing the virus normal guinea pig serum was added to a final concentration of 5 per cent (7) upon which inactivation at 37 C for one hour was carried out This suspension constituted the final antigen which was stored in small rubber stoppered vials at —20° C. Before use the antigen was thawed and centrifugated at 2000 r p m for 10 minutes

Torso suspensions from normal mice of comparable age were prepared by the same method and used as antigen controls

The final CF antigens were ordinarily waterclear sometimes of a slightly yellowish colour Prolonged storage at —20° C resulted in an increased amount of sediment after the last centrifugation

[illegible] CF [illegible]

The details regarding complement sheep red cells haemolysin veronal saline diluent and guinea pig serum employed in the present experiments were the same as those described previously (4)

## EXPERIMENTAL AND RESULTS

### *Box Titrations of Prototype Antigens*

To determine the potency of the CF antigens and the CF antibody titer of the hyperimmune sera, box titrations", *i e* titrations involving varying serum dilutions as well as varying antigen dilutions, were performed Serial twofold dilutions of antigens and hyperimmune sera were prepared in veronal buffer Tubes containing the serum, complement and antigen dilutions (0 1 ml of each added to the tubes in the order mentioned) were incubated at 4°C overnight, *i e* for 18 to 20 hours The next morning 0 2 ml of sensitized sheep red cells were added to each tube and haemolysis was allowed to take place in a water bath at 37°C for 30 minutes Subsequently the tubes were placed at 4°C, and the results were read the following morning Complete fixation was recorded as 4, no fixation as 0, and intermediate degress as 3, 2, 1, +, and (+) A fixation of 3 or 4 was ordinarily employed for re- [illegible] endpoint of the titration, the titers of the antigens and

In a forthcoming publication (5) the results obtained with a number of freshly isolated virus strains will be reported

## MATERIALS AND METHODS

*Experimental animals* Suckling albino mice usually less than 24 hours and never more than 48 hours of age were used for virus titrations as well as for preparation of antigens Animals used for Coxsackie B studies were always less than 24 hours old To obtain an even distribution of the animals all of the suckling mice in an experiment were pooled—as is the usual procedure in this laboratory—and subsequently distributed at random seven youngs to each mother In the following a group of seven such mice will be referred to as a litter

For hyperimmunization purposes mice weighing 22 28 g were used

*Virus strains* The Coxsackie virus strains used for preparation of prototype reference antigens were our laboratory strains comprising various mouse passages of all of the available prototype Coxsackie strains Of these the strains A 1 through 19 and B 2 through 5 had been kindly supplied by *Dr G Dalldorf* strain B 1 by *Dr J L Melnick*, the strains A 20 through 24 by *Dr Hildegard Plager* In addition group A antigens of the types 2 5 12 and 15 were prepared using as starting material mouse passages of strains of these types received from *Dr Hildegard Plager* Furthermore CF antigens were prepared fr [illegible] echo 9 strain kindly supplied by [illegible] tissue culture passage of a [illegible] from the first mouse passage of a strain of herpes simplex virus kindly supplied [illegible] *Dr K Birkum Petersen* of this institute representing the seventh passage in HeLa cells

*Virus titrations* Serial ten fold dilutions were inoculated intraperitoneally into newborn mice using one litter per dilution The inoculum was 0 02 ml The mice were observed daily for seven days for paralysis or other typical signs of disease and *Karbers* method (6) was used in the calculation of endpoints

*Hyperimmune sera* were prepared according to a method described in detail in an earlier paper (4) The technique [illegible] mately 30 mice were employed T [illegible]

The virus suspensions used for inoculation were prepared from torsos of newborn mice infected with our laboratory Coxsackie virus strains The fluids were subjected [illegible] which in this study was used for hyperimmunization [illegible] sometimes observed [illegible] by fluorocarbon[1] treatment using the method to be described for the CF antigen preparation

The sera were stored undiluted at 20° C Before use in CF tests they were thawed diluted in veronal buffer usually 1 4 and inactivated at 60° C for 20 minutes When serum pools or low dilutions of sera were prepared in amounts sufficient for several experiments they were stored at —20° C during the experimental period

*Titration of virus neutralizing antibodies in hyperimmune sera* were carried out on most of the hyperimmune mouse sera employed The serum was diluted 1 4 in saline and inactivated at 60° C for 20 minutes Serial two fold dilutions were now made covering a supposedly adequate interval and to 0 2 ml of each dilution was added 0 2 ml of a dilution of the homologous virus type containing about 200 $ID_{50}$ (80 800 $ID_{50}$) pr 0 02 ml The serum virus mixtures were incubated for one hour at room temperature and subsequently inoculated into newborn mice For each

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[1] *Fluorocarbon* (Freon 113 (Trichlorotrifluoroethane CCl₂F CClF₂ E I Du Pont de Nemours & Company Inc ) was employed in the present experiments

serum preparations a brief comment on the results shall be given here. It will be noted that although the same batch of complement – stored in sealed ampoules at –20°C – was used in the three experiments, small variations could be seen in the fixation grades obtained in the complement titrations in veronal buffer made at different times and using different ampoules of complement. The results of the control titrations of complement furthermore reveal that exactly two units of complement were not always available in all test tubes. Usually approximately two units of complement were present in the tubes with the lowest dilutions of antigens and sera, more than two, sometimes a little more than three units, were not infrequent in the tubes containing the higher dilutions of antigens and sera.

TABLE 2

*Box Titration Representative for Reactions Characterized as "Medium"*
Coxsackie A 23. Expt. No. 369, Nov. 10, 1960

| | Homologous hyperimmune serum | | | | | | | | | | | | Antigen control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 | 4096 | 8192 | |
| Antigen undil. 1 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 2 | + | (+) | (+) | (+) | 0 |
| 2 | 4 | 4 | 4 | 4 | 4 | 4 | 2 | 1 | (+) | 0 | 0 | 0 | 0 |
| 4 | 3 | 3 | 3 | 2 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| 8 | 1 | 1 | 1 | 1 | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Serum control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Control titration of complement

| | Complement | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
| Antigen undil. | 0 | (+) | (+) | + | + | 1 | 1 | 1 | 1 |
| 2 | 0 | 0 | (+) | + | + | + | 1 | 1 | 1 |
| 4 | 0 | 0 | (+) | (+) | + | + | 1 | 1 | 1 |
| 8 | 0 | 0 | 0 | + | + | + | 1 | 1 | 1 |
| 16 | 0 | 0 | 0 | (+) | + | + | 1 | 1 | 1 |
| Serum 4 | 0 | 0 | 0 | + | + | 1 | 1 | 1 | 1 |
| Serum 8 | 0 | 0 | 0 | (+) | + | + | 1 | 1 | 1 |
| Complement control | 0 | 0 | (+) | + | + | + | 1 | 1 | 1 |

Legends: see Table 1.
Same batch of complement as employed in Expt. No. 362, Table 1. Complement dilution 1:15 was used in the box titration.

It will be seen that generally the antigens were slightly anticomplementary if used undiluted, occasionally if used 1:2. The higher dilutions of antigens rarely showed anticomplementary effect, and even in the presence of undiluted antigen the fixation obtained in the complement titrations with one unit of complement seldom exceeded 1, the reaction usually being 0 or (+). As can be seen from the complement titrations in the presence of hyperimmune sera, these might be more or less anticomplementary in their lowest dilutions. However, the

sera being recorded as the highest dilution of antigen or serum giving 3 or 4 fixation In some titrations where the maximal fixation was 2 1 or +, this fixation was used for calculating the endpoint (see later) Appropriate controls of antigen and serum as well as a cell control and a control titration of complement with and without the antigen dilutions employed, often also with the highest serum concentrations employed, were included in each box titration experiment

TABLE 1

*Box Titration Representative for Reactions Characterized as Strong*

Coxsackie A 5 Expt No 362 Nov 2 1960

| | Homologous hyperimmune serum | | | | | | | | | | | | Antigen control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4* | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 | 4096 | 8192 | |
| Antigen undil | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 2 | 1 | + | (+) | (+) | 0 |
| 2* | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 1 | (+) | 0 | 0 | 0 |
| 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 3 | 1 | 0 | 0 | 0 | 0 |
| 8 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 1 | (+) | (+) | 0 | 0 | 0 |
| 16 | 2 | 2 | 2 | 2 | 2 | 2 | 1 | + | 0 | 0 | 0 | 0 | 0 |
| 32 | 0 | 0 | 1 | 1 | + | + | (+) | (+) | 0 | 0 | 0 | 0 | 0 |
| Serum control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Control titration of complement §

| | Complement | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20* | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
| Antigen undil | 0 | (+) | + | 1 | 1 | 1 | 1 | 1 | 1 |
| 2* | 0 | (+) | (+) | + | + | 1 | 1 | 1 | 1 |
| 4 | 0 | (+) | (+) | + | + | + | 1 | 1 | 1 |
| 8 | 0 | (+) | (+) | + | + | 1 | 1 | 1 | 1 |
| 16 | 0 | 0 | (+) | + | + | 1 | 1 | 1 | 1 |
| 32 | 0 | 0 | (+) | + | + | 1 | 1 | 1 | 1 |
| Serum 4 | 0 | 0 | + | 1 | 1 | 1 | 3 | 2 | 2 |
| Serum 8 | 0 | 0 | + | 1 | 1 | 1 | 2 | 2 | 2 |
| Complement control | 0 | (+) | (+) | + | – | + | 1 | 1 | 1 |

* *Reciprocal of dilutions*

§ *Complement dilution 1 15 was used in the box titration*

In Tables 1, 2, and 3 are presented the results of typical experiments The data shown in Table 1 are representative for the fixation grades obtained with some of the Coxsackie types which yielded CF antigens of high potency and hyperimmune sera with a considerable CF antibody titer Such reactions have been characterized as strong Tables 2 and 3 show representative results obtained with other Coxsackie types, yielding reagents the reactions of which in box titrations could be characterized as "medium" or "weak"

In the three tables are included the simultaneous control titrations of complement with and without dilutions of antigen and serum and since these titrations are representative for the results obtained in a great number of titrations carried out with the diffe

11, 13, 17, 18, 19, and 21 were less or not at all satisfactory for routine purposes

As regards the Coxsackie B viruses the tests for types 1, 2, and 3 showed so low fixation grades that they in the table are characterized as "unreliable". Coxsackie viruses B4 and B5 gave completely negative results Thus it can be concluded that employing the present technique, Coxsackie B antigens are not suitable for routine CF tests

TABLE 4

*Box Titrations Performed with the Various Coxsackie Virus Types*

| Virus | Number of tests | Number of antigen preparations | Number of serum batches | Representative test No | Antigen titer | Serum titer | General characterization of CF reactions |
|---|---|---|---|---|---|---|---|
| Coxs A 1 | 4 | 2 | 2 | 312 | 2* | 128* | weak to medium |
| 2 | 6 | 4 | 4 | 361 | 2 | 512 | medium |
| 3 | 5 | 2 | 2 | 337 | 8 | 512 | strong |
| 4 | 4 | 2 | 2 | 323 | 8 | 1024 | strong |
| 5 | 6 | 3 | 4 | 362 | 8 | 512 | strong |
| 6 | 4 | 2 | 2 | 338 | 8 | 512 | strong |
| 7 | 3 | 1 | 2 | 322 | 4 | 256 | medium |
| 8 | 4 | 2 | 1 | 324 | 4 | 256 | medium |
| 9 | 3 | 1 | 2 | 325 | 2 | 64 | weak to medium |
| 10 | 3 | 2 | 1 | 328 | 2 | 256 | medium |
| 11 | 4 | 2 | 1 | 372 | 1 (2) | 32 (2) | unreliable |
| 12 | 6 | 3 | 4 | 330 | 8 | 1024 | strong |
| 13 | 4 | 2 | 2 | 331 | 1 (+) | 64 (+) | unreliable |
| 14 | 3 | 2 | 2 | 332 | 4 | 512 | medium to strong |
| 15 | 5 | 2 | 4 | 290 | 2 | 128 | medium |
| 16 | 4 | 2 | 2 | 236 | 1 | 64 | weak |
| 17 | 4 | 2 | 2 | 237 | 1 (1) | 128 (1) | unreliable |
| 18 | 3 | 2 | 2 | 238 | 1 (1) | 64 (1) | unreliable |
| 19 | 5 | 2 | 2 | 239 | 1 (2) | 32 (2) | unreliable |
| 20 | 2 | 1 | 1 | 365 | 2 | 512 | medium |
| 21 | 3 | 2 | 1 | 390 | | no fixation | negative |
| 22 | 3 | 1 | 1 | 391 | 2 | 64 | weak to medium |
| 23 | 2 | 1 | 1 | 369 | 4 | 256 | medium |
| 24 | 2 | 1 | 1 | 370 | 1 | 128 | weak |
| B 1 | 3 | 2 | 2 | 295 | 1 (1) | 32 (1) | unreliable |
| 2 | 3 | 2 | 1 | 392 | 1 (+) | 64 (+) | unreliable |
| 3 | 3 | 2 | 2 | 371 | 1 (+) | 256 (+) | unreliable |
| 4 | 4 | 2 | 1 | 384 | – | no fixation | negative |
| 5 | 3 | 1 | 2 | 393 | – | no fixation | negative |

* Reciprocal of highest dilutions showing 3 or 4 fixation Where maximal fixation obtained was less than 3 the fixation grade is added in brackets

Conversely, tissue culture fluids of monkey kidney cells infected with Coxsackie B strains give appreciable fixation with homologous mouse hyperimmune sera (3) and with homologous hamster hyperimmune sera (8) An experiment was therefore made with Coxsackie B1 through 5 and with A9, in which both tissue culture antigens[1] and

[1] These antigens were kindly made available by *Dr Annelise Gottfredsen* of this Institute

anticomplementary effect of final antigens and of hyperimmune sera has never been of such a magnitude as to make the interpretation of results of the box titrations difficult. Reactions resembling prozone phenomena were seen occasionally although they were not very pronounced and did not occur with any consistency.

TABLE 3

*Box Titration Representative for Reactions Characterized as "Weak"*
Coxsackie A 24 Expt No 370, Nov 10 1960

| | Homologous hyperimmune serum | | | | | | | | | | | | Antigen control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 | 4096 | 8192 | |
| Antigen undil | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 1 | 1 | 1 | 0 | 0 | 0 |
| 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | + | + | 0 | 0 | 0 |
| – 4 | 0 | + | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| – 8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Serum control | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*Control titration of complement*

| | Complement | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
| Antigen undil | 0 | + | + | 1 | 1 | 1 | 1 | 1 | 1 |
| – 2 | 0 | 0 | (+) | (+) | + | + | 1 | 1 | 1 |
| – 4 | 0 | 0 | 0 | (+) | (+) | + | 1 | 1 | 1 |
| – 8 | 0 | 0 | 0 | 0 | (+) | + | + | 1 | 1 |
| Serum 4 | 0 | 0 | (+) | (+) | + | 1 | 1 | 1 | 1 |
| Serum 8 | 0 | 0 | (+) | (+) | + | 1 | 1 | 1 | 1 |
| Complement Control | 0 | 0 | 0 | (+) | + | + | 1 | 1 | 1 |

Legends see Tables 1 and 2

For the majority of the virus types several batches of antigen and hyperimmune serum were prepared and examined during the study. Table 4 presents a survey of the results obtained in box titrations in which 24 Coxsackie A and 5 Coxsackie B strains were tested against the homologous sera. For each strain the total number of tests is recorded together with the number of antigen preparations examined and the number of hyperimmune serum batches tested. For each virus type two to six box titrations were performed and one experiment typical of the strain has been given (No 312 for Coxsackie A1, No 361 for Coxsackie A2 etc.). If the maximal fixation was only 2, 1, or +, these fixation grades are added in brackets. The table also includes a general characterization of the reactions obtained with the various virus types. The term "unreliable" indicates that the reaction has been found weak and not easily reproducible.

The preparations and test methods worked satisfactorily for 18 out of the 24 Coxsackie A strains. However, the results obtained with types

periments performed in which all prototype antigens were tested against seven pools of Coxsackie hyperimmune sera The antigens were employed undiluted and diluted 1 2 yet antigens which in box titrations had been found to give weak or no fixation with homologous sera together with the two antigens prepared from herpes simplex and echo 9 virus strains were tested undiluted only Serum pools I to VI contained the Coxsackie A hyperimmune sera in the following combinations Pool I A 1–4 Pool II A 5 8 Pool III A 9 12 Pool IV A 13 16 Pool V A 17–20 Pool VI A 21 24 Serum Pool B contained Coxsackie hyperimmune sera B 1 through 5

The final dilutions of the individual sera contained in the serum pools were generally 4 or 8 times lower than the dilution determined in box titration as the CF antibody titer of the serum However to simplify the preparation of the pools and to avoid potential non specific reactions no serum was employed in a final dilution lower than 1 16 the dilutions of individual sera in the pool containing the Coxsackie B sera were 1 20

It will be seen from Table 6 that the majority of the Coxsackie antigens were found to react with the homologous serum pool and with this pool exclusively However the reactions obtained with antigens of the strains A 9 A 11 and A 24 were weak and no fixation at all occurred with antigens of the A strains 13 17 18 19 21 and the B strains 1 through 5 It should be mentioned that in other experiments with slightly less complement the reactions on the whole have been stronger and weak specific reactions have been observed with antigens A 17 and A 19 However it has been a consistent observation that no or only minimal fixation was encountered between antigens A 13 A 18 and B 1 through 5 and their homologous antisera and this has been the case even when lower dilutions of the sera have been employed Antigen A 21 has been tested only once against all of the heterologous antisera

Furthermore from Table 6 it will be seen that neither the two control antigens nor the antigen prepared from a herpes simplex virus strain showed any reactions with the serum pools whereas the antigen prepared from the mouse pathogenic echo 9 virus strain reacted with serum pool VI containing Coxsackie hyperimmune sera A 21 through 24 When echo 9 antigen was tested against the individual sera in the pool it was demonstrated that the antigen reacted with Coxsackie A 23 antiserum and with this serum exclusively

In the experiment recorded in Table 6 minimal crossing was found only between antigen A 3 and serum pool II containing hyperimmune sera A 5 through 8 In other experiments comprising testing of all of the antigens undiluted and diluted 1 2 against the individual hyperimmune sera contained in the reacting pools definite two-way crossings have been observed between the antigens and sera of the strains A 5 and A 12 occasionally a slight crossing has been found between A 11

mouse antigens were tested against the homologous mouse hyperimmune sera. It will be seen from Table 5 that good fixation was obtained with all of the Coxsackie B tissue culture antigens, and as regards types 1, 2 and 3 reactions were definitely stronger than those obtained with mouse tissue antigens. The two Coxsackie A9 antigens did not differ in quality.

TABLE 5

*Testing of Mouse Tissue CF Antigens and Tissue Culture CF Antigens in Comparative Box Titrations*

Coxsackie A 9 and B 1 through 5

| Virus | Mouse tissue antigens | | Tissue culture antigens | |
|---|---|---|---|---|
| | Serum titer | Antigen titer | Serum titer | Antigen titer |
| Coxsackie A 9 | 64* | 1* | 32* | 1* |
| B 1 | 32 (1) | 1 (1) | 32 | 1 |
| – 2 | 64 (+) | 1 (+) | 16 | 1 |
| – 3§ | 256 (+) | 1 (+) | 128 | 1 |
| 4 | no fixation | | 32 | 1 |
| – 5§ | no fixation | – | 32 | 1 |

* Reciprocal of highest dilutions showing 3 or 4 fixation in box titrations. Where maximal fixation obtained was less than 3, the fixation grade is added in brackets.
§ Inoculum used for hyperimmunization was purified by fluorocarbon treatment.

### *Studies on Type Specificity of the Prototype Antigens*

To determine the extent of heterologous cross-reactions all of the prototype CF antigens were tested against all of the prototype hyperimmune sera.

Since both herpes simplex virus and echo 9 virus can be recovered from stool specimens, and both may give signs of disease in newborn mice, clinically resembling those produced by Coxsackie viruses, antigens prepared in the usual way from mice diseased after inoculation of herpes simplex virus and a mouse pathogenic echo 9 virus strain, respectively, were also included in these experiments.

The CF technique employed in these studies was the same as the one described for titration of the prototype antigens. As described previously the anticomplementary effect of the antigens and sera employed was of such low order as to leave unaffected the interpretation of results obtained in the CF tests. Consequently, complement titration in the presence of antigens and sera were not included in this series of experiments. Fixation grades of 3 or 4 were considered positive reactions. Whether fixation grades of 2 or less should be considered as specific was determined by comparison with the results obtained with the other specific reagents and with the control reagents included in the tests.

In Table 6 are presented the results obtained in one of several ex-

*Notes to Table 6*

Simultaneous complement titration in Veronal buffer

| Units of complement | 2 | 1 5 | 1 | 0 75 | 0 5 |
|---|---|---|---|---|---|
| Fixation grade | 0 | 0 | 0 | 0 | (+) |

* 1 = undiluted 2 = diluted 1 2

§ Dilutions of the individual sera in the pools 4 or 8 times lower than the CF antibody titer of the sera yet at least 1 16 Dilution of each individual serum in pool B 1 20

† Prepared from normal suckling mice two days old

|| Prepared from normal suckling mice four days old

and A 15 One-way crossing has been encountered consistently between antigen A 3 and hyperimmune serum A 8 The degree of these reactions has varied according to the dilutions of sera employed It has been found most pronounced in experiments in which undiluted antigens were tested against hyperimmune sera in dilutions as low as 1 5 or 1 8

However, when sera have been employed in the dilutions listed in Table 6, no or only slight crossings between the types just mentioned have been observed, and the Coxsackie antigens have been found to react with strong fixation with the homologous hyperimmune serum exclusively

## DISCUSSION

The results obtained in the present series of CF experiments with all of the prototype Coxsackie virus strains are in agreement with the preliminary experiments (4)

Antigens prepared from 18 of the 24 Coxsackie A types examined, were found to give satisfactory complement fixation, while the five Coxsackie B viruses examined gave less reproducible results or no complement fixation at all

For the majority of the virus types several batches of antigen and hyperimmune serum were prepared and two to six box titrations were carried out with each virus type This was done in order to compare in successive experiments the same preparations of antigen and serum and to study preparations made at different times When the same batches of antigen and serum were employed in successive box titrations the titers of the reagents usually did not vary more than two fold When the same antigen was titrated against different homologous hyperimmune sera, or when antigens prepared at different times were titrated against the same batch of hyperimmune serum, the variations

ually were not more than
eight fold Finally, when
which batches of antigen
as well as serum varied the titers of antigen and serum obtained might vary up to eight-fold Therefore it should be stressed that the antigen- and serum titers recorded in Table 4 are the results of one experiment chosen as representative for each virus type, while the characterization

TABLE 6

*Cross Testing of the Prototype CF Antigens against 7 Different Pools of Coxsackie Hyperimmune Sera*

| Antigen |  | Hyperimmune serum pools |  |  |  |  |  |  | Normal mouse serum | Antigen control | Dilutions [illegible] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Virus | Dilution | I A 1-4 | II A 5-8 | III A 9-12 | IV A 13-16 | V A 17-20 | VI A 21-24 | B B 1-5 |  |  |  |
| Coxsackie A 1 | 1* | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
|  | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| – – 2 | 1 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 64 |
|  | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| – 3 | 1 | 4 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 128 |
|  | 2 | 3 | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| – – 4 | 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 128 |
|  | 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| – 5 | 1 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 128 |
|  | 2 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| – 6 | 1 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 128 |
|  | 2 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| 7 | 1 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 32 |
|  | 2 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| 8 | 1 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 32 |
|  | 2 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| 9 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
|  | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| 10 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
|  | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| 11 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
| – 12 | 1 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 128 |
|  | 2 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| – 13 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
| 14 | 1 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 64 |
|  | 2 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 |  |
| – 15 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 32 |
|  | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |  |
| 16 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 16 |
|  | 2 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |  |
| – 17 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
| 18 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
| – 19 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
| – – 20 | 1 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 64 |
|  | 2 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |  |
| 21 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 16 |
| 22 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 16 |
|  | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |  |
| – 23 | 1 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 32 |
|  | 2 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 |  |
| – 24 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 16 |
|  | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| B 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 |
| – 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 |
| 3 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 |
| – 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 |
| – – 5 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 |
| Echo 9 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 |  |
| Herpes simplex | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| Control I† | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
|  | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| Control II‡ | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
|  | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |
| Serum control | – | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |  |

Notes see next page

Among the A antigens with lower infectious titers, some displayed medium CF activity, for instance A 2, A 15 and A 20, while others showed weak or no CF activity. Types 11, 13 and 16 are examples of this.

Regardless of the level of the infectious titers none of the Coxsackie B antigens here examined gave reliable CF reactions.

TABLE 8

*Corresponding CF and Virus Neutralizing Antibody Titers of the Various Types of Coxsackie Hyperimmune Sera*

| Serum | | Titer of CF antibodies | Titer of virus neutral antibodies | Number of $ID_{50}$ in neutral test |
|---|---|---|---|---|
| Coxsackie A | 1 | 128* | 1024§ | 80 |
| – | 2 | 512 | 8192 | 450 |
| | 3 | 512 | 8192 | 60 |
| | 4 | 1024 | 8192 | 60 |
| | 5 | 512 | 4096 | 100 |
| | 6 | 512 | 4096 | 250 |
| – | 7 | 256 | 1024 | 100 |
| | 8 | 256 | 1024 | 60 |
| | 9 | 64 | 64 | 100 |
| | 10 | 256 | 1024 | 200 |
| | 11 | 32 (2) | 1024 | 60 |
| | 12 | 1024 | 4096 | 80 |
| | 13 | 64 (+) | 512 | 40 |
| | 14 | 512 | 2048 | 400 |
| | 15 | 256 | 2048 | 25 |
| | 16 | 32 | 1024 | 40 |
| | 17 | 128 (1) | 512 | 60 |
| | 18 | no fixation | 1024 | 300 |
| | 19 | 16 (2) | 256 | 40 |
| | 20 | 512 | 2048 | 30 |
| | 21 | no fixation | 32 | 125 |
| | 22 | 64 | 1024 | 25 |
| | 23 | 256 | 2048 | 25 |
| | 24 | 128 | 1024 | 100 |
| B | 1 | 32 (1) | 512 | 160 |
| | 2 | 64 (+) | 128 | 150 |
| | 3 | 256 (+) | 1024 | 60 |
| | 4 | no fixation | 256 | 25 |
| | 5 | no fixation | n d † | |

* Reciprocal of highest serum dilution showing 3 or 4 fixation in box titrations, where maximal fixation obtained was less than 3 the fixation grade is added in brackets

§ Reciprocal of highest initial serum dilution giving 100 % protection of the inoculated mice

† n d = not done

As mentioned previously the infectious titers of the CF antigens in most experiments are derived from only one titration in baby mice. Experiments in this laboratory (3) have shown that when a Coxsackie virus suspension stored at —20°C is titrated in newborn mice on different days or in two experiments on the same day, the variation in titer

"strong", "medium" or "weak" is based on several experiments. Reactions characterized as "unreliable" could not regularly be reproduced.

The potency of the antigens prepared from different virus types varied considerably. In order to see whether the infectious titer of an antigen was correlated to its CF antigenic potency, the infectious titer of each final antigen was determined, usually by only one titration in newborn mice. The infectious titers of the "representative" antigen preparations referred to in Table 4 have been listed in Table 7. Due to cannibalism amongst the mice the titration endpoints of a few of the antigens were not very distinct. In the table this has been indicated with a "ca".

Comparison with Table 4 will show that Coxsackie A antigens with a high infectious titer, such as the types 3, 4, 5, 6, 8, 10, and 12, always displayed strong or medium CF activity. Type A 1 was found to be an exception to this general rule. Also in other laboratories (9) this virus type has been found to give irregular results in several respects.

TABLE 7

*Comparative Infectious Titers of Prototype Coxsackie CF Antigens and of Coxsackie Stock Virus Suspensions*

| Virus | Infectious titer* of CF antigen | Average infectious titer* of stock virus | Number of infectivity titrations of stock virus |
|---|---|---|---|
| Coxsackie A 1 | ca 7.1 | 7.5 | 9 |
| 2 | ca 6.5 | 7.7 | 9 |
| 3 | 7.9 | 7.8 | 4 |
| 4 | 8.1 | 8.1 | 6 |
| 5 | 8.9 | 8.2 | 6 |
| 6 | 7.5 | 8.0 | [illegible] |
| 7 | ca 6.0 | 5.5 | 6 |
| 8 | ca 7.4 | 7.7 | 6 |
| 9 | 5.7 | 5.6 | 6 |
| 10 | 7.7 | 8.0 | 4 |
| 11 | 5.4 | 5.3 | 4 |
| 12 | 8.2 | 7.8 | 8 |
| 13 | 5.6 | 6.2 | 5 |
| 14 | 6.7 | 6.6 | 5 |
| 15 | 5.8 | 6.0 | 6 |
| 16 | 5.6 | 6.2 | 5 |
| 17 | 5.4 | 5.2 | 5 |
| 18 | 5.8 | 5.6 | 4 |
| 19 | 5.5 | 5.7 | 6 |
| 20 | 4.1 | 4.4 | 6 |
| 21 | 4.1 | 4.5 | 4 |
| 22 | 6.5 | 6.5 | 5 |
| 23 | 6.4 | 6.0 | 4 |
| 24 | 6.0 | 5.8 | 3 |
| B 1 | 6.5 | 6.3 | 11 |
| 2 | 5.1 | 4.6 | 4 |
| 3 | 6.4 | 6.1 | 8 |
| 4 | 5.1 | 5.2 | 8 |
| 5 | n.d. | ca 3.4 | 5 |

* Neg. log $ID_{50}$. Other legends: see text.

moderate, reciprocal cross reactivity of these types has been found in neutralization tests in newborn mice (11) On the other hand in the present study CF cross reactions were not observed between the virus strains A 13 and A 18 and between A 8 and A 12, as have also been found in neutralization tests (11) As far as A 13 and A 18 are concerned this might be due to a too low CF activity of the reagents

The antigen prepared from an echo 9 virus strain isolated in this laboratory reacted in the cross CF tests with serum prepared from Coxsackie A 23 This latter virus strain is now generally considered identical with echo 9 virus and has been proposed removed from the Coxsackie group

As regards cross reactions it shall be added that in the CF tests some presumably non specific reactions between various serum pools and some of the antigens were seen repeatedly when the reagents were tested undiluted or in low dilutions

Finally, it should be mentioned that several of the mice, hyperimmunized with Coxsackie B 1 and B 3 viruses died after having shown signs of illness similar to those described by *Pappenheimer et al* (12) A few of the mice being hyperimmunized with Coxsackie A 1 and A 24 viruses displayed flaccid paralysis of the hind legs a finding in accordance with observations made in other laboratories (13 14)


## SUMMARY

Complement fixing (CF) antigens from the prototype strains of 24 Coxsackie A viruses and 5 Coxsackie B viruses were prepared by a method involving fluorocarbon treatment of infected torso suspensions of newborn mice (4) The antigens were examined in CF tests against homologous and heterologous hyperimmune mouse sera

In the Coxsackie A group 18 out of 24 antigens gave satisfactory complement fixation while the remaining six Coxsackie A strains as well as the Coxsackie B strains gave less reproducible results or no fixation at all Apart from a few well defined minor crossings, all reacting antigens were found to be type specific

The correlation between the level of infectious titers and CF potency of the antigens and between the level of CF- and antibody titers of hyperimmune mouse sera is discussed


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3 *Staut* [illegible]
4 *Staut* [illegible] [illegible]aration of complement fixing antigens for routine typing of Coxsackie viruses Acta path et microbiol scandinav *50* 55–63 1960

may occasionally be as high as 1.6 log units. In Table 7 is therefore listed also the "average" infectious titer of all of the stock virus batches based on four to eleven infectivity titrations in suckling mice performed from time to time. It will be noted that a rather good correlation was found between the infectious titers of the final CF antigens and of the stock viruses. This indicates that the purification of the CF antigen including treatment with "Freon 113" does not essentially affect the infectious titers of the virus suspensions. Coxsackie A 2 is most probably an exception, since the infectious titer of three other A 2 antigen preparations have also been reduced. Summarily it may be concluded that a Coxsackie virus suspension with an infectious titer of $10^{-6.5}$ or more consistently yielded a reliable CF antigen, whereas infectious titers lower than about $10^{-6.5}$ apparently have failed to present any consistent correlation between the size of the infectious titer and the CF potency in the strains used. Other strains of the same serotypes may be more suitable *for a preparation of CF antigens, as has been observed in cases of other viruses (8, 10).*

That even high infectious titers of the initial material may fail to result in reliable CF activity of Coxsackie B mouse tissue antigens is apparent from the present study as well as from results obtained previously (4).

Two of the hyperimmune sera, B 3 and B 5 had been prepared by inoculation of virus suspensions purified by "Freon 113"-treatment. It can be seen (Table 5) that the use of such inocula resulted in antisera with good complement fixing qualities.

No comparisons between mouse tissue antigens and tissue culture antigens were attempted with the Coxsackie A strains which showed minimal or no fixation in the present experiments.

In Table 8 the CF and neutralizing antibody titers are compared for one batch of each type of hyperimmune serum. As regards the group A viruses a certain correlation between these titers seem to exist since a high level of CF antibodies always corresponds to a high level of virus neutralizing antibodies. However, when sera are titrated against the present CF antigens even a considerable titer of virus neutralizing antibodies is not always reflected in a high titer of CF antibodies, as indicated by the results from tests with sera A 11, A 13, A 17 and A 18.

Amongst the results listed in Table 8 it is noteworthy that, although the inocula used for hyperimmunization had satisfactory infectious titers, the virus neutralizing antibody titers of sera A 9 and A 21 were very low as compared to the other types. Moreover Coxsackie virus A 9 consistently gave irregular results in neutralization tests.

The cross reactions demonstrated in CF tests between the Coxsackie strains A 5 and A 13, and between A 3 and 8 are in accordance with results published by other investigators (2). The slight two-way crossing occasionally observed between strains A 11 and A 15, has – as far as we know – not been reported by others. However, definite, although

moderate reciprocal cross reactivity of these types has been found in neutralization tests in newborn mice (11) On the other hand in the present study CF cross reactions were not observed between the virus strains A 13 and A 18 and between A 8 and A 12 as have also been found in neutralization tests (11) As far as A 13 and A 18 are concerned this might be due to a too low CF activity of the reagents

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3 *Sla t Munch B*

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# BRIEF REPORT

## SERUM DISAPPEARANCE RATE OF GAMMA GLOBULIN IN RHEUMATOID ARTHRITIS [1]

By K. Aho, K. Sievers, K. Simons and O. Wager

The rheumatoid factor (RF) behaves in many respects like an antibody against human gamma globulin. As the antigen antibody complexes usually are eliminated from the serum more rapidly than the antigen alone, it seems possible that the serum disappearance rate of gamma globulin might be shortened in patients with high titers of RF. The results hitherto reported on the gamma globulin disappearance rate in rheumatoid arthritis have been somewhat conflicting (1, 3, 5, 6).

TABLE

*Serum Disappearance Rate of $I^{131}$ Labelled Gamma Globulin*

| Patient | Age Years | Diagnosis | Waaler Rose Titer | Disappearance Rate Day [1] |
|---|---|---|---|---|
| K U | 60 | Rheumatoid arthritis | 2000 | 0.059 |
| L L | 66 | Rheumatoid arthritis | 2000 | 0.048 |
| H K | 51 | Rheumatoid arthritis | 1000 | 0.043 |
| L H | 55 | Rheumatoid arthritis | 1000 | 0.066 |
| K T | 40 | Rheumatoid arthritis | 1000 | 0.054 |
| I K | 57 | Rheumatoid arthritis | 1000 | 0.075 |
| K M | 51 | Rheumatoid arthritis | 500 | 0.064 |
| E N | 60 | Rheumatoid arthritis | 500 | 0.046 |
| A R | 47 | Rheumatoid arthritis | 500 | 0.051 |
| F K | 38 | Rheumatoid arthritis | 500 | 0.074 |
| E K | 52 | Rheumatoid arthritis | 500 | 0.061 |
| S K | 22 | Rheumatoid arthritis | 0 | 0.044 |
| B T | 21 | Rheumatoid arthritis [1] | 0 | 0.071 |
| S M | 56 | Rheumatoid arthritis | 0 | 0.051 |
| A R | 61 | Rheumatoid arthritis | 0 | 0.039 |
| A O | 48 | Rheumatoid arthritis | 0 | 0.064 |
| R R | 53 | Rheumatoid spondylitis | 0 | 0.073 |
| L H | 23 | Rheumatoid spondylitis | 0 | 0.085 |
| T M | 52 | Rheumatoid spondylitis | 0 | 0.055 |
| H A | 40 | Osteoarthritis | 0 | 0.040 |

[1] The patient had also renal amyloidosis

For the present study 20 patients were selected among 300 patients with rheumatic disease up for treatment ... the Rh...
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graph...

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Factor h serum contains antibodies to three antigens $h_1$ $h_2$ and $a_2$. The presence of strong antibodies to $n$ requires further absorption of the $h$ serum with strain 3647 (cf (1)). This is in accordance with Grun's (4) suggestion of a common antibody in the $b$ and $h$ sera. The $h_2$ antigen is heat stable but the agglutination is blocked both in autoclaved and live cultures. Agglutination with mannitol salt agar cultures grown at 20°C and 37°C reveals this antigen (Table 1) and has also revealed several other antigens

TABLE 1

*Agglutinability of Type Strains in $h_2$ Serum*

| Culture | Strain | | | | | |
|---|---|---|---|---|---|---|
| | 1503 | 28 | 365 | 17 A | Cowan I | Cowan II |
| Nutrient agar live and autoclaved bacteria | — | — | — | — | — | — |
| Mannitol salt agar 37°C | — | — | — | + | ++ | ++ |
| Mannitol salt agar 20°C | ++ | ++ | ++ | +++ | ++ | ++ |

The $h$ serum is prepared from serum 17 A by absorption with 17 A nutrient agar cultures — No agglutination + to +++ Strength of reaction

Two strains 670 and 5687 were found to possess unblocked $h_2$ agglutinogens in nutrient agar cultures Strain 5687 has no $h_1$ antigen and is thus suitable for preparation of the $h_1$ serum

Factor $i$ serum As reported before (5) this serum contains strong antibodies to the $m$ antigen It is to be expected that also antibodies to $c$ are present The $i$ serum is therefore now prepared from serum F 21 by absorption with strains 1503 and 3467 Two antibodies to factors and [illegible] this serum An $i_2$ serum is produced by [illegible] while a pure $i_1$ serum cannot be prepared

Factor $k$ serum The $k$ serum ha[illegible] anti bodies to $k$ and $m$ Antibod[illegible] The resultant se[illegible] and $k_3$ Antibod[illegible] tively The refer[illegible] technique but s[illegible] salt agar cultures are used

The $m$ serum prepared from serum F 21 contains *additional antibodies to* $i_2$. We have not yet found a strain possessing the $i_2$ antigen and not the $m$ antigen

The $f$ and $g$ sera have not been used by us for years and no new antibodies have been found in $e$ and $n$ sera. It is at present not clear whether any of the new antigens described by *Grün* (2 3) are identical to the antigens reported here

Four new antigens have been detected in the strains Cowan I and II and two new a[illegible]

The [illegible] without further comm[illegible]

*References* 1 *Cohen J O & Oeding P* J Bact., in press 1962—2 *Grun L* Ztschr Hyg *145* 259 1958 3 *Grün L* Zentralbl Bakt Orig I *181* 210 1961—*Grün L* Ztschr Hyg *148* 345 1962—*Haukenes G & Oeding P* Acta path et microbiol scandinav *49* 237 1960—6 *Oeding P* Acta path et microbiol scandinav Suppl 93 356 1952

filtration (Sephadex G-25) The product contained an average of two atoms of iodine per molecule of gamma globulin In immunoelectrophoresis it showed only a single line

Eleven of the patients had rheumatoid arthritis having high titers of RF in their sera and showing positive one tube latex test Five patients with rheumatoid arthritis and all the three patients with rheumatoid spondylitis had completely negative Waaler-Rose and latex tests In one of the patients with seronegative rheumatoid arthritis (B T ) the disease was complicated by renal amyloidosis One patient had osteoarthritis of the hip

Blood samples were taken during the first week every second day and later on twice a week The radioactivity was counted in a well type scintillation detector After an initial rapid decline of serum radioactivity during the equilibration phase the decrease followed an exponential curve after the third day The serum disappearance rate of injected gamma globulin was calculated from the data of the subsequent two weeks' period The results are shown in the table During the following weeks the disappearance rates appeared to be somewhat slower possibly due to inherent heterogeneity and different catabolic rates of injected purified gamma globulin However the small amount of radioactivity retained did not permit accurate calculations

It is seen in the table
with the disappearance
with osteoarthritis all
it was not possible to
different phases of the disease process

After completion of the present experiments results similar to ours were reported by *Olhagen et al* (4)

*References* 1 *Grubb R* Acta haemat 20 246 1958 —2 *McFarlane A S* Nature 182 53 1958 —3 *Mills J A Calcins F & Cohen A S* J Clin Invest 40 1926 1961 —4 *Olhagen B Birke G Liljendahl S O* ... IX Scandinavian Congress of Rheumatology Coper
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## SEROLOGICAL TYPING OF STAPHYLOCOCCUS AUREUS

By *Gunnar Haukenes*

*A thorough study of one of Oeding's (6) factor sera the c serum led to the* detection of two new antigens (5) The other factor sera have now been examined by the same technique Since the results have great consequence for the serological typing a preliminary report will be given

The investigations have primarily been based upon the absorbing capacity of type strains and new methods for revealing blocked agglutinogens

The factor sera *a b* and *c* were found to have the following antibody composition

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Factor *a* serum | $a_1$ | $a$ | $a_3$ | $a_4$ | $a_5$ | | | | |
| Factor *b* serum | | $a$ | $a_3$ | $a_4$ | $a_5$ | $b_1$ | $b_2$ | $b_3$ | |
| Factor *c* serum | | $a_2$ | | $a_4$ | $a_5$ | $b_1$ | | | $c_1$ |

The antigens $a_5$ and $c_1$ are heat stable Factor sera to $a_1$ $a_4$ $a_5$ $b_1$ $b_2$ and $c_1$ have been prepared

Received 4 vii 62 *from the University of Bergen School of Medicine the Gade Institute Department of Microbiology Bergen Norway*

results have been compared with those obtained with Stuart s transport medium Among others 90 gonococcal strains were investigated and the TMG showed better viability results than Stuart s medium Excellent results were obtained with haemolytic streptococci pneumococci Haemophilus influenzae and with anaerobic non spore forming bacteria Improved results were also demonstrated when small inocula were employed and when bacterial cells which had previously been affected by growth inhibitory conditions were cultured Microorganisms which were inoculated from the TMG medium in comparison with those from other tested transport media showed a shortened lag phase

In order to increase the storage capacity and safety in transport cocoa fat with a small addition of vaseline can be layered on the medium Such a seal which is essential with low agar concentration and or a large volume of the medium can be easily melted over a flame before inoculation and thereafter permitted to harden on the surface of the transport medium For the majority of the bacteria tested the viability was best maintained at +24° C while the gonococci survived better at +10° C

*Møller O* Bacteriological Department Borås SOME TECHNICAL ASPECTS ON THE LÖWENSTEIN CULTURE

A simple and reliable method of preparing and sterilizing the Löwenstein medium is described The ingredients are mixed in the ordinary way, but no sterile precautions are necessary The medium is tubed and coagulated in the conventional manner whereafter the tubes are autoclaved at 110–120° C for 30 or 15 minutes

For decontamination of the specimens a modification of Saxholm s method has given very satisfactory results The concentration is somewhat lower (0 5 per cent desogen 0 25 per cent pancreatin) and the contact time 18 hours (over night) at +4° C After inoculation of the medium the decontamination tubes are kept for two weeks at +4° C If inspection of the cultures during the first weeks shows growth of irrelevant bacteria new Löwenstein tubes are inoculated from the decontamination tubes By this method it has been posible to obtain pure positive cultures of tubercle bacilli from *a priori* heavily contaminated specimens

Prolongation of the incubation time of the Löwenstein cultures from 6 weeks to 3 months has in a material of 1660 cultures with 140 positives resulted in two (2) additional positive cultures In both cases the guinea pig inoculation had given a negative result

*Lind A & Norlin Margareta* The Department of Bacteriology University of Gothenburg Gothenburg OXYGEN CONSUMPTION IN SAUTON CULTURES OF ANONYMOUS MYCOBACTERIA

The standardization of the antigens is one of the problems when trying to perform a serological differentiation between various species types and strains of mycobacteria The intention of this preliminary study was to investigate whether or not measurement of the oxygen consumption of the mycobacterial cultures might be of value in this connection

Eight strains representing the four groups of Runyon were examined In the course of nine weeks the oxygen consumption was measured at weekly intervals with the Warburg technique Each strain was investigated in duplicate series and the results were found to be fairly reproducible On the other hand different species

# TRANSACTIONS
# OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

*Meeting May 6, 1962*

at

*The Institutes for Medical Microbiology Gothenburg Sweden*

Received 28 v 62

*Nygren B* The Department of Clinical Bacteriology University of Gothenburg Gothenburg CONTINUED STUDIES ON CLOSTRIDIUM PERFRINGENS AND BACILLUS CEREUS AS CAUSES OF FOOD POISONING

Food poisonings caused by Cl perfringens and B cereus have a quite similar symptomatology Both of these microbes have the ability to produce a *lecithin* splitting enzyme of identical type phospholipase C Since these two common qualities seemed rather striking they directed the suspicion towards the product of the enzymatic reaction phosphorylcholine as being the cause of the illness The accuracy of this hypotheses has been strengthened by demonstration of this substances in naturally and experimentally infected material and by experiments of its intestinal effect on animals Some conditions for and variations of the production and activity of the phospholipase C enzyme have also been considered

*Heinertz N O* Department of Bacteriology University of Gothenburg Gothenburg
AN HOSPITAL INTOXICATION BY B CEREUS OF A SOMEWHAT UNUSUAL CHARACTER

*Moller A* Department of Bacteriology University of Gothenburg Gothenburg
A NEW TRANSPORT MEDIUM FOR BACTERIOLOGICAL SAMPLES

A transport medium should be bacteriostatic and must be capable of maintaining cell viability stand lengthy storage and have an indicator which shows when it is no longer suitable for use It should be easily handled and transported as well as reproducible on a large production scale The maintenance of viability and bacteriostatic effect are of great importance and difficult to achieve An attempt to satisfy the aforementioned requirements has been made with a new transport medium (*TMG* [1])

The medium is prepared with balanced quantities of inorganic and buffering salts reducing substances and organic material in agar gel The bacteriostatic effect is provided by an addition of phenylmercuric acetate The quality of the ingredients including the distilled water and agar has proved to be of great importance

The medium has been tested with a number of common bacterial strains and the

---

1 *Transport Medium* of the Bacteriologic Laboratories of *Gothenburg*

*Berglund G* Department of Bacteriology, University of Gothenburg Gothenburg

IMMUNOELECTROPHORETIC STUDIES ON FIBRINOGENOLYSIS AND FIBRINOLYSIS

Fibrinogen plasmin and the lytic products of fibrinogen and fibrin were investigated immunoelectrophoretically employing corresponding antisera produced in rabbits

The fibrinogen was shown to have a changed mobility according to the degree of dilution The plasmin could not be freed of plasminogen The plasmin plasminogen precipitate formed with anti plasminogen was located in the $\beta_2$ globulin region

The lytic products of long term fibrinogenolysis consisted of at least 2 antigenic factors which were related to fibrinogen The lytic products of fibrin contained two similar factors as well as an additional one

By employing anti plasminogen to decrease the plasminogen content in the fibrinogen preparation a changed course of the fibrinogenolysis was obtained Thus the importance of the quantity of the plasmin to the reaction was demonstrated

of one and the same Runyon group had oxygen consumption curves showing various trends

The development of precipitinogens in the culture filtrates was studied and compared with oxygen consumption the pH variation and colour intensity

*The results were discussed*

*Hedlund P Lycke E & Tibblin G* The Medical Clinic I and the Municipal Virological Laboratory, Sahlgren's Hospital Gothenburg THE ASSOCIATION OF ACUTE BENIGN PERICARDITIS IN ADULTS WITH COXSACKIE B 5 VIRUS INFECTION

*The clinical findings* from six patients diagnosed as acute benign pericarditis are presented Five of these were adults The viral studies showed that the patients were COXSACKIE B 5 excretors and developed homologous antibody titers in serum A rise in antibody titer was demonstrable in three cases In the other three cases high antibody levels were already found on admittance but these patients had been ill for more than two weeks

Although available materials are small they demonstrate the association of acute benign pericarditis and Coxsackie B virus infections In the present investigation the viral studies indicated Coxsackie B 5 virus as the etiological agent The isolation of virus from the pericardial fluid which would support the demonstration of the etiology was not attempted The isolation of Caxsackie B 3 virus from the pericardial fluid as well as from stool specimens has been reported by *Kagan & Bernkopf (Am Ped 189 49 1957)* in a ten month old girl with pericarditis

Two of our patients showed leucocyte counts of more than 9 000 per $mm^3$ and elevated antistreptolysin O titers were observed in three cases Beta haemolytic streptococci were found in throat swabs from the six year old boy Therefore the possibility that two of the patients also had bacterial complications cannot be disregarded

The frequency of pericarditis during epidemics of Coxsackie B viruses is probably higher than indicated by available reports The disease has been observed mainly among children Five of the six cases in the present report however were adult patients and two were admitted to hospital under a preliminary diagnosis of heart infarction

*Lund Ebba* The virological Laboratory of the Department of Bacteriology University of Gothenburg and the Virus Department of the Municipal laboratories Gothenburg OXIDATIVE INACTIVATION OF POLIOVIRUS

A functional relationship between the rate of oxidative inactivation of poliovirus and the oxidation potential was found when oxidants such as chlorine chloramine permanganate and ions of heavy metals were used The dependency of the rate of inactivation on pH temperature and the concentration of the reactants has been studied using roller tube cultures of trypsinized monkey kidney cells

The oxidation potential is dependent just as much on the quantity and character of the reducing components present as on the added oxidant The rate of inactivation of poliovirus by means of free or combined available chlorine shows the same dependency on the potential irrespective of the purity of the virussuspension The amount of free or combined chlorine necessary to give a certain potential is dependent however on the purity of the virussuspension

*Berglund G* Department of Bacteriology University of Gothenburg Gothenburg

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# ANALYSES – REVIEWS – REFERATE

*Atlas of Avian Hematology* Alfred M. Lucas, A B, Ph D, Cytologist and Casimir Jamroz, B S, A B, Medical Illustrator. 271 pages. 413 figures and 24 tables USDA Monograph 25 U.S Government Printing Office, Washington 1961 Price $4 50

This monograph constitutes an outstanding piece of work and should greatly help bring order into a confused and somewhat neglected field The title does not do justice to its content because it is more than an atlas in the usual sense of the word where the text is a mere description of the illustrations Instead, the entire field of morphological haematology in the avian species is covered in a superbly styled interpretative, and highly informative text The illustrations, which are almost exclusively in colour, are of the highest possible quality

A short review can not give full credit to the importance and excellence of the atlas The emphasis is on fowl haematology but a special chapter is devoted to other pertinent avian species A most valuable feature is that all illustrations on normal haematopoiesis come from normal individuals and not from pathologic specimens as is the case in many other atlases The most controversial issue is the terminology that Dr Lucas has adopted Current terminology, which is based on human haematopoietic cells has been considered inadequate for non mammalian species Dr Lucas has, therefore, introduced some different concepts and terms based on his observations of avian cells The reasons for this are convincing and very clearly elaborated From this work it does not seem unlikely that the present anthropocentric terminology will eventually submit to a terminology similar or equal to that here proposed With more knowledge of comparative haematology, it should be possible to work out a suitable terminology applicable to all vertebrates

The monograph describes the appearance of haematopoietic cells from embryos of as early as 46–47 hours of incubation It contains very valuable sections on technical procedures including methods for obtaining blood samples from embryos Normal values for different strains and ages of fowl and other avian species have been tabulated Special attention has been given to the physiological variation encountered among the cells There should no longer be any excuse for describing normal cell features as pathologic

The volume is not only invaluable to anyone dealing with avian haematology but is of great interest to those working with mammalian haematology who have an excellent opportunity of broadening and deepening their general knowledge of blood cells

*Jan Pontén*

FROM THE PATHOLOGICAL DEPARTMENT (HEAD O GRØNTOFT M D), CENTRAL HOSPITAL VÄNERSBORG SWEDEN

# RHABDOMYOMA AND RHABDOMYOMATOSIS OF THE HEART

By

THORBJØRN BERGE

Received 19 iii 62

*Von Recklinghausen* (2), in 1862, was the first to describe a case of rhabdomyoma in the heart Up to 1955, 77 cases have been recorded (31), since then and up to the end of 1960 the author has found in the literature another 10 cases (3, 20, 26, 6, 30, 12, 4, 1, 7, 9) Thus, together with the case to be discussed here, a total of 88 cases of rhabdomyoma has been reported

So far the tumour has been diagnosed clinically only once (X-ray diagnosis, verified at operation) (12) More than 50 per cent of the patients die before attaining one year of age (2)

The first case in a negro was described in 1935 (14), subsequently, further cases have been reported

In view of the fact that a rhabdomyoma frequently occurs concurrently with a number of different malformations it is now the general opinion that it is not a real tumour, but a malformation-tumour, a hamartoma

## CASE HISTORY

The second child of a 29 year old woman II pregnancy Mother and infant RH negative W R negative Borne at full time, weight 3630 gr Nothing noteworthy on examination after delivery Subsequently, difficulty in breathing developed and clinically an increasing enlargement of the heart was seen Despite treatment with digitalis the infant died when it was nine days old with symptoms of heart insufficiency

*Macroscopic description* The heart was greatly enlarged weight 60 gr and showed concentric hypertrophy and considerable dilatation on the left side Both of the atria were dilated The ostia of the pulmonic artery and aorta were extensively stenosed so that only a fine slate pencil could pass the [illegible]

[illegible]

ac [illegible]

fo [illegible]

m [illegible] cut surface In this region the heart muscle measured 2½ cm (see Fig 1) Otherwise the heart muscle was homogenous The endocardium of the left ventricle had thickened very considerably it was of an ashen grey colour but smooth everywhere Acute stasis was observed in the liver spleen and kidneys No data of interest were obtained from the brain

# ANALYSES – REVIEWS – REFERATE

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The volume is not only invaluable to anyone dealing with avian haematology but is of great interest to those working with mammalian haematology who have an excellent opportunity of broadening and deepening their general knowledge of blood cells

*Jan Ponten*

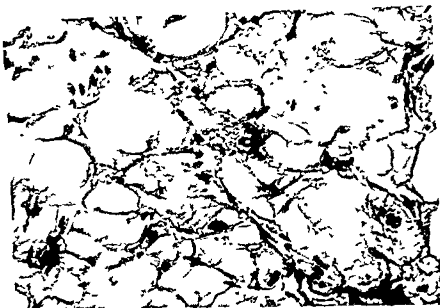


*Fig 3*
Spidercell in a rhabdomyoma (van Gieson × 380)

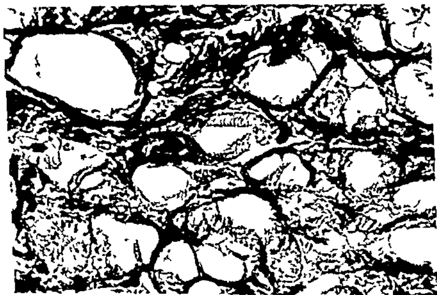


*Fig 4*
Transverse striation by rows of delicate granules (Heidenhahn × 608)



*Fig 1*
Rhabdomyoma in anterior wall of left ventricel and fibroelastosis of the endocardium (× 2)

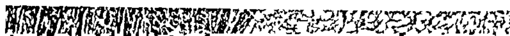


*Fig 2*
To the right the typical vacuolization in a rhabdomyoma To the left ordinary myocardium (van Gieson × 38)

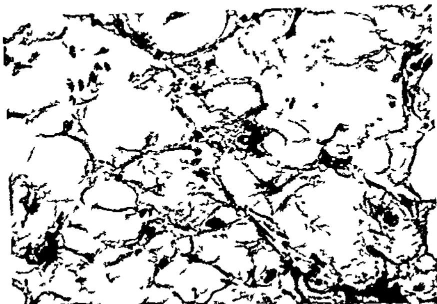
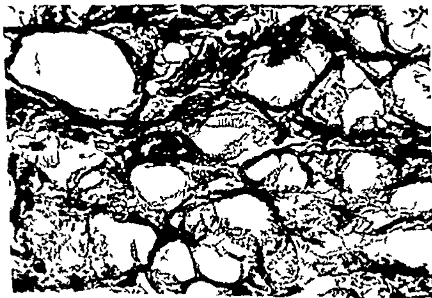


*Fig 3*
Sp lercell in a rhabd myoma (van C eson × 380)



*F g 4*
Transver e striati n b v r ws of lel cate granu les (He lenhahn × 608)

*Microscopic investigation* The tumour in the left ventricle was rich in cells and strongly vacuolated (see Fig 2) In the centre of the vacuoles there were frequently polygonal cells with radiate processes (spider cells) Both the cytoplasm and the processes *display fine-grained transverse striations in sections stained according to Heidenhahn* (see Figs 3 and 4) With Best's carmine stain glycogen was demonstrated in sections which were pretreated with absolute alcohol only and mounted directly on glass without a water bath being used The tumour is sharply demarcated from the surrounding muscles but is without a proper capsule In its immediate surroundings and in a number of places below the endocardium small rhabdomyomatous foci are present the histologic picture of which varies to a certain degree representing all the transitions from a normal myocardium to the picture of the tumour described above No spider cells occur in the foci, but a presence of glycogen is demonstrable All of the foci are localized compactly below the endocardium The endocardium has thickened and is built up of connective tissue rich in collagen and containing an abundance of elastic fibres

*Pathological anatomical diagnosis* Rhabdomyoma et rhabdomyomatosis cordis + Stenosis valvulae pulmonalis et aortae + Hypertrophia et dilatatio cordis + Fibroelastosis endocardii

## DISCUSSION

Benign tumours which proceed from the striated muscles are rarely encountered Some authors doubt the existence of such tumours and consider that the rhabdomyomas described are malformations According to *Winston Ewans* (32) no actual rhabdomyoma has been observed either in the skeletal musculature or in the heart

In a material consisting of 400,000 sections primary cardiac tumours represented only 0 017 per cent of the total number of tumours (29)

Rhabdomyomas are described as occurring either in a diffuse form or as single or multiple nodules (8) The characteristic, large, empty spaces within the tumour have been interpreted as lymph space, artifacts, etc *Wohlbach* (33) was the first to succeed in demonstrating, with the aid of a special stain, that these empty spaces were localized within the muscle-fibres *Rehder* (25) showed that their contents were made up of glycogen, a fact which had previously been suggested by *Seiffert* (27)

There are various theories as to the genesis of rhabdomyomas On account of morphologic similarities and its glycogen content, rhabdomyomas were assumed to originate from Purkinje's cells *Steinbiss* (28) showed however, that rhabdomyomas could be found in regions distant from Purkinje's cells and that disturbances in nervous transmission never were demonstrated On the whole these tumours affect the heart only when they cause disturbances in the closing mechanism of the valves The tumours show no marked tendency to proliferate (25)

Most investigators now regard rhabdomyomas as tumours due to malformation, i e as hamartomas (25, 21, 17, 13) This is indicated by the fact that the typical spider cells resemble the heart musculature at a certain stage of development, and that the tumours display only a slight tendency to proliferate Moreover, frequently other concurrent malformations are present, *e g* mixed tumours of the kidneys (10), adenomatous hyperplasia of the sweat glands in the skin (16), cystic kidneys

18), developmental disturbances in the heart and aorta, aortic hyperplasia, atrial defects, and most common of all, tuberous sclerosis, which has been found to occur in 50–60 per cent of the cases (5, 25, 18)

When von Gierke (11) described his disease, hepatonephromegalia glycogenica, he mentioned a case of diffuse hyperplasia of the heart musculature, as a consequence of infiltration with glycogen *Olsen & Cooper* (22) consider that the glycogen stratifications described in connection with rhabdomyomas may all have been local manifestations of von Gierke's disease *Pompe* (23) is of the opinion that some of the cases reported as idiopathic cardiac hypertrophy were cases of von Gierke's disease

The above-mentioned malformations or tuberous sclerosis are not found as frequently, however, in association with von Gierke's disease Other conditions as well make it probable that it is a question of two different diseases The glycogen in rhabdomyomas is more soluble in ordinary fixing fluids than is the glycogen present in von Gierke's disease where its presence still can be demonstrated after a number of weeks and months By staining and mounting the preparation without employing a water bath according to *Beaird* (3), we were able also to demonstrate the presence of glycogen, when staining in the usual way had proved unsuccessful

In order to show that they are not actual tumours it has been proposed to use the term "congenital nodular glycogenic tumour of the heart (2) and "congenital nodular glycogenic degeneration of the myocardium" (22)

It is our view also that we are dealing here with a tumour due to malformation This is indicated by its combination with other malformations (pulmonary and aortic stenosis), and the histologic picture showing the presence in the heart not only of a delimited tumour but also of multiple rhabdomyomatous foci at different stages of development, and finally, by the glycogen only being slightly stainable


SUMMARY

A case of so-called rhabdomyoma and rhabdomyatosis of the heart is described At the autopsy of a mature male child, who had died from cardiac insufficiency at the age of nine days, findings in the myocardium included multiple rhabdomyomatous foci of [illegible]

[illegible] glycogen present in von Gierke's disease Furthermore pulmonary and aortic stenosis was observed No data of interest were found in the other organs The poor stainability of the glycogen and the absence of lesions in the liver, spleen, and kidneys probably exclude von Gierke's disease The histologic picture,

and the other concurrent malformations support the view that rhabdomyomas are tumours of malformations type, so called hamartomas

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# CORRELATION OF TENSILE STRENGTH AND CHEMICAL COMPOSITION IN EXPERIMENTAL GRANULOMA

*By*

J. Viljanto and E. Kulonen

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The experimental granuloma has been the subject of numerous chemical studies (for general references see *Woessner & Boucek*, 1961) but it has not been possible to compare directly the tensile strength of the recently formed collagenous fibres with the chemical composition of the granulation tissue. From a healing wound the granulation tissue is difficult to prepare quantitatively for chemical study from the adjoining original tissue and it has not been possible to estimate the tensile strength of whole pieces of experimental granulomata.

The emphasis of the present investigation was on the direct comparison of the chemical and mechanical properties of the same granulation tissue. The principle was to use pieces of suitable implantation material, which had been divided into two halves but fixed again in original position with stitches. After the implantation period in subcutaneous space of the rat, the experimental granuloma could be separated clearly from the surrounding tissue. The stitches were removed and the tensile strength measured by drawing the two halves apart. The second point of emphasis was to study the immediate period after implantation, *i.e.*, the "lag phase" before the collagen synthesis begins. Into this methodical paper we included also the effect of immersion of the implantation materials into solution of soluble collagen, and into solution of carrageenin, which has frequently been used to provoke the formation of experimental granulomata (*Robertson & Schwartz*, 1953).

## EXPERIMENTAL

*Animals.* Wistar rats (about 100 g) were used. At this age the necessary space for the implantation material could be prepared with a minimal traumatization of

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the sub utane us tissue In later experiments it was foun l that the age and the eight of the an mals s mewhat influence the results The animals were kept in v re net cages usually 2 in each

*Techn que of implantat on* The animals were anaesthetized with ether The l aved sk n v as cleaned with ethanol and about 3 cm long medial incision was l nc on the back caudally of the scapula The space between fascia and skin (with ubcutaneous fat) was opene l w th l lunt en led forceps and two implants were pla c l symmetrically The inc sion was cl sed with ster l zed cotton varn

*Implantat on mater als* In the earlier exper ments Sponcal" sp nge (visc se cllul se) tampons (standard pieces of ab ut 40 × 10 × 10 mm wet manufact ıred f r the dental practice by Svenska Cellulosa Ab Sundsvall Sver ge) were used The p eces of the range 170 190 mg were used but cut 20 mm long Later we use l p eces of v sc se cellulose ponge Visella (prepared for us by Saters Oy Ltd Valkeak ki Finland) The sulphur had l een removed by washing the pieces l l a hed and finall treated w th glycerol to soften them One cm³ of dry Visella ponge we ghe l 74 mg The average p re size ( open cell system") was al ut 0 2 mm diameter and the coml ne l surface area f the pores about 60 cm²/cm³ sponge M st f the present exper ments were made with Sponcal" sponge only in the regranulat on experiments Visella sp nge was used However the Visella" ponge was adopted t the standar l use As far we know the bas c material is the am both in Sp ncal and in Visella l ut in the latter the pore size is smaller wh ch is an advantage The granulat on tissue grows into it eas ly better than into Sp n al tampons We appreciate the advantages of the viscose cellulose as im plantat n material it is rather h mogenous easy to cut and stitch inert and thus u tal le for mechan cal and chem cal study sterilizable by dry heat or boiling and easily impregnable with desired s lutions

The pieces 20 × 10 × 10 mm were cut with scissors in tw halves wh ch were sewn t gether w th 4 stitches They were steril zed in bo l ng water f r 15 min

In n n m n he [illegible] olution (in Tampereen [illegible] the carra [illegible] ume of 0 9

p r cer Na l solut n The viscous solut on was clarif ed by centrifugation (20 m n 4300 r p m

In an add ti nal er es the implanted material was s ake l w h [illegible]

[illegible]

re regranulati n experiment the p eces were f rst implanted for 5 days taken ut an l the halv s lrawn apart without removing the through g ing varns wh h kept the halves t gether Then the halves were allowed to take the original l t n an l the pieces were impl ante l aga n f r further 7 days after which the t nsile strength v as mea ure l

*Me surement of the tens le strength* The tensile strength was measured with a tr p le beam balance n minal sens tiv ty 1 mg After removing the stitches the impl ante l d ubl piece was suspended t one side f the balance from the upper half an l the l wer half of the samp le was fixed A l eaker was put on the other side f the l alance This side of the balance was now charged by allow ng water to flow 300 ml m n ) nto the beaker until on the other s de the halves of the implanted p ng br ke apart The we ght of the water was recorded The sk n wounds were t st l im larly fr m l em br ad ste ps excise l perpend cularly to the wound

*Chem cal letern n it r s* f r chem cal determinati ns the implanted sp nges w r lr l f r 4 lays at +80 C weighe l an l homogenized w th "Buhler" hom g n z r l 25 f ll v v lume f flui l at full spee l (n minally 50 000 r p m ) Th llagen was gelat n zed in aut clave (2 atm 2 hrs with water in neutral pH) and f ltered thr ugh s ntered glass (corresp nding to Jena 11 C 4) which retained all the ins l l le cellul e It was ob erved that when implantati n lasted longer than 2 days some c llagen (less than 10 per cent of the total) was still present in the residue an l ha l t be measure l separately Total nitrogen was analyzed by

Kjeldahl combustion and subsequent destillation of ammonia. The collagen content was calculated from the hydroxyproline content determined according to *Neuman & Logan* (1950).

The samples for nucleic acid determinations were stored at —15° C. To 0.2 ml of the tissue homogenate (in 0.9 per cent NaCl) 0.2 ml of 10 per cent trichloroacetic acid solution was added and heated 15 min. at +90° C. The suspension was diluted with 2 ml of 5 per cent trichloroacetic acid. The mixture was filtered and the precipitate washed with 2 ml of 5 per cent trichloroacetic acid. The ultraviolet light absorption was measured from the combined supernatant at 2600 Å with Beckman *DU* spectrophotometer against reagent blank solutions. The total amount of nucleic aci[illegible] d fo[illegible] D-oxy[illegible] lan[illegible] tot[illegible] of [illegible] stu[illegible] *Big*[illegible] be too high because of the error caused by protein impurities.

## RESULTS

*Morphological description.* The paraffin blocks were sectioned at 10 μ and stained with haematoxylin-eosin and Heidenhain's azan. After 24 hrs. implantation the sponge was surrounded by a thin capsule (staining faintly blue with azan) and a zone of mononuclear leukocytes. The cells invaded to the center of the sponge during the second day. On the third day the first elongated fibroblasts and erythrocytes were observed, but distinct capillaries first on the fourth day. More fibroblast "islets" appeared and their outgrowths stained pale blue with azan. The fibrin retired gradually. From the seventh day onwards the fibroblasts increased, filled the entire sponge and the azan staining turned more intense. However, the change in the histological structure did not clearly reflect the abrupt increase of the tensile strength and collagen (Fig. 2). Histological pictures of the sponge granulomata are published in other context (*Viljanto & Kulonen* 1962).

*DNA, RNA and total nitrogen.* The data (Fig. 1) show in agreement with *Williamson & Guschlbauer* (1961) that 2—5 times RNA is produced in comparison to DNA, which latter reaches the maximum at 7th day. After the very first days a temporary decrease in RNA is noted. The total nitrogen increased earlier than collagen. We believe that this initial increase of nitrogen is not primarily due to increased intracellular protein but to imbibition of the pieces with tissue fluid and from 3 days onwards with blood carried through the developing capillaries (cf. *Jackson* 1958).

*Tensile strength and amount of collagen.* Fig. 2 summarizes the results. About 2—3 days after implantation there is a first slight maximum in collagen content. From the 5th day onwards the main collagen synthesis begins, which is parallelled with the development of the tensile strength.

*The effect of carrageenin.* In Table 1 it is shown that the soaking of the sponge in carrageenin does not have any beneficial effect on the

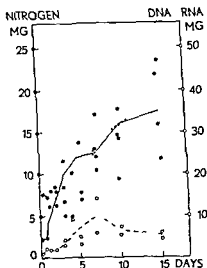



*Fig 1*

*DNA ( ○ – ○ – ○ – ) RNA ( ⊷ ⊷ ⊷ ) and total nitrogen (—● ●—) per piece of Sponcal granuloma tissue*

The lines are fitted visually. At the time range 6–48 hrs eight granuloma pieces were homogenized together into one sample. At later time points the granulomata from 4 5 rats were combined to one homogenate. Each point represents one homogenate

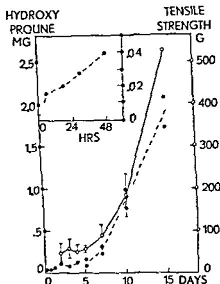



*Fig 2*

*Tensile strength (—○—○—○— with standard deviation) and hydroxyproline (– ● –●––●––) per piece of Sponcal granuloma tissue*

The hydroxyproline content during the first 48 hrs is shown in the insert. Each point of hydroxyproline represents one homogenate of eight granuloma pieces from 4 rats

TABLE 1

*Effect of Carrageenin on the Tensile Strength of Granuloma Tissue*

| Days implanted | No. of implants | Average tensile strength g piece | | |
|---|---|---|---|---|
| | | Control | Carrageenin | Carrageenin / Control × 100 |
| 2 | 2 | 40 2 | 20 9 | 51 9 |
| 3 | 3 | 39 4 | 33 7 | 85 5 |
| 4 | 3 | 41 9 | 43 5 | 103.8 |
| 5 | 3 | 56 3 | 35 9 | 63 7 |
| 7 | 3 | 42 4 | 34 5 | 81.3 |
| 15 | 2 | 129 7 | 106 1 | 81 8 |
| | | | | Average 78 0 ± 18 |

tensile strength of the granulation tissue. Carrageenin accordingly acts as a foreign body only and seems not to have any specific stimulating effect on the collagenous tissue. This finding is at variance with the experiments of *Bush & Alexander* (1960) who used cotton pellets as implantation material and enchanged the granuloma formation by

soaking the implants with carrageenin solution. With cotton pellets we found some benefit from carrageenin 4 days after implantation on the weight of the granulation tissue. The sample of carrageenin had been used successfully in the production of granulomata. In some experiments, not described in detail, the fractions of carrageenin (prepared according to *Smith, O'Neill & Perlin*, 1955) were tried (without sponge) and the λ-fraction was more active than κ-fraction, but the difference was not marked.

TABLE 2
*Effect of Neutral Salt-Soluble Collagen on the Tensile Strength of Experimental Granulation Tissue*

| Days implanted | Tensile strength g/piece | |
|---|---|---|
| | Control implants | Implants imbibed with soluble collagen |
| 1 | 8.6 | 11 |
| 2 | 40 | 61 |
| 3 | 47 | 58 |
| 5 | 49 | 62 |
| 7 | 73 | 83 |

Each figure is the average of three determinations. The difference between the groups is statistically significant ($P < 0.005$) when tested as non-independent pairs.

TABLE 3
*Tensile Strength of Experimental Granulation Tissue and Skin Wounds after Preliminary Implantation of the Viscose Cellulose Pieces*

| Experiment | Tensile strength (g) on ... th day | |
|---|---|---|
| | Granuloma | Skin wound |
| After 5 days preimplantation | 86 ± 19 (4) | 350 ± 96 (5) |
| Controls | 48 ± 25 (5) | 201 ± 69 (8) |

The standard deviation is indicated. Number of measurements in the parentheses.

*Effect of soluble collagen and "regranulation".* Table 2 shows that the tensile strength was increased when the implants had been impregnated beforehand with soluble rat collagen ($P < 0.005$). If granulation tissue is present in the sponge already on the "zero day", the tensile strength develops much stronger at continued implantation than without preexisting granulation tissue (Table 3).

## DISCUSSION

This procedure has certain advantages. (1) The tensile strength can be measured on a well defined granulation tissue, which is grown in (2) sterile conditions. This arrangement is decidedly better for the production of granulation tissue than open skin wounds, which are often infected and their granulation tissue is not easily delimited. (3) The

volume of the granulation tissue is kept constant at least during the early phase. An advantage over turpentine abscesses is that (4) the irritation of the surrounding tissue is minimal. There is also a (5) possibility to imbibition of the granulation tissue with desired solutions. However it was observed that the granulation tissue develops stronger near the surface of the sponge and therefore it is important that the circumference of the cross sectioned area (of contact between the halves) is constant and not too small. Perhaps better results would be obtained with a cross sectional area of a parallelogram. The standard deviations of various groups in average 8 measurements were calculated in per cent and these standard deviations in the tensile strengths of Visella granulomata were in the range of 4.8–35.4 per cent (17 groups, average 22 per cent), in skin wounds 5.8–33.3 per cent (17 groups, average 24 per cent).

The origin of the collagen during the first days is obscure. Either a cellular source may be taken into consideration or it may be suggested that the imbibing tissue fluid contains some soluble collagen. At the next phase the amount of collagen rather decreases during the proliferation and maturation of the fibroblasts before the final main collagen synthesis begins. The next step of this investigation is the elucidation of the factors which determine the consequent details in collagen synthesis, amino acids, soluble collagens, maturation to insoluble fibres and the balance with simultaneous decomposition of collagen.

The rather modest effect of soluble collagen could be explained most easily by a clot formation. It has been found by several workers that fibrin clot inside the sponge implant enchanges the formation of granulation tissue (e.g. *Edwards, Sarmenta & Hass* 1960). Thus it is not necessary to consider any specific effect of added soluble collagen on the formation of fibres. *Peacock* (1961) made an extensive study on the effect of the collagenous extracts on wound healing. He got positive results with acid extracted (but neutralized) collagen solutions only. He concludes that "if collagen from external source has any opportunity to participate in the formation of new scar tissue it will probably have to be introduced as a monomeric form."

From the Table 3 it is obvious that when a ready granulation tissue is present the lag phase before the collagen fibre formation is shortened. It is not known whether intact granulation tissue is necessary or whether e.g. homogenate would be sufficient. The faster healing of a resutured wound has been often cited in the literature (for general reference see *Walker* 1961).

## SUMMARY

The growth of granulation tissue into viscose cellulose sponge was studied with emphasis (1) on the comparison of the tensile strength and chemical composition of the granulation tissue and (2) on the lag phase immediately after implantation.

Collagen is observed in experimental granulomata in small scale in few hours after implantation, and significantly from the fifth day onwards. The tensile strength runs approximately parallel.

*Soaking of the implanted material with soluble collagen increased the* tensile strength of the granulation tissue. Imbibition with carrageenin slightly decreased the tensile strength of cellulose sponge granulomata.

*The total nitrogen in the implanted sponges increased very rapidly,* presumably through the imbibition with tissue fluids and blood. Preliminary data are presented on the formation of DNA and RNA.

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*FROM THE DEPARTMENT OF PATHOLOGY SÖDERSJUKHUSET STOCKHOLM SWEDEN (HEAD F WAHLGREN MD)*

# SUBCUTANEOUS GRANULOMATA FOLLOWING INOCULATION OF INFLUENZA VACCINE

*By*

S R ORELL

Received 13 ix 62

During recent years a number of small subcutaneous lesions have been submitted to the pathological laboratory at the Sodersjukhuset for histological examination They were all situated on the upper arms of otherwise healthy patients and revealed a characteristic histological picture They consisted for the greater part of sclerotic, collagenous tissue Centrally there were large acidophilic necroses which were finely granular or almost completely structureless and of a fibrinoid like appearance Fibrin staining by the Weigert or Ladewig method proved negative however Silver impregnation revealed that the reticulin fibres were also destroyed It was thus a question of coagulation necrosis The necroses often contained rounded hyaline bodies which assumed a pale rose colour after van Gieson staining Sometimes there was also calcification In a few cases the necroses were sparsely edged with fibroblasts sometimes with a palisade like arrangement while the surrounding connective tissue was sparsely infiltrated with lymphocytes In other cases the process had a fresher appearance The connective tissue around the necroses then contained numerous swollen fibroblasts and macrophages and a fairly profuse infiltration of lymphocytes plasma cells and eosinophilic leucocytes Occasionally groups of foreign body giant cells were also observed Finally in most cases there were dense infiltrations of lymphocytes in the boundary zone to the surrounding fat tissue often in the form of lymph follicles with evident germinal centres (Fig 1)

The number of subcutaneous granulomata of the type described which so far have been observed amounts to 15 from 15 patients 6 of these were sent to us from other hospitals 10 patients were female and 5 male The ages varied between 18 and 64 The patients sought medical advice after noticing a hard often painful nodule on the upper arm In no instance was there either fistulation or suppuration The nodules were excised under local anesthesia and the healing was rapid

I wish to thank *Prof C Olin* and *Dr L Heller* Statens Bakteriologiska laboratorium Stockholm and *Dr C Wallmark* Dept of Bacteriology Sodersjukhuset Stockholm for their help and stimulating interest in this work

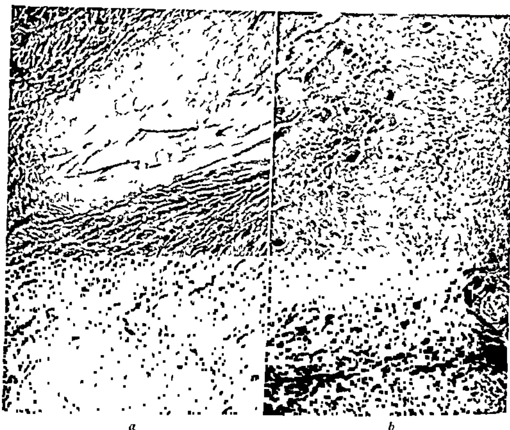


*Fig 1*

a) Subcutaneous granuloma excised 16 months after inoculation of aluminium oxide adsorbed influenza vaccine Note finely granular necrosis (top) and peripheral lymphocytic infiltration with follicle formation (bottom) van Gieson × 60

b) Subcutaneous granuloma excised 12 months after inoculation of aluminium oxide absorbed influenza vaccine Note palisade-like arrangement of fibroblasts edging necrosis (right) van Gieson × 60

The position of the nodule on the outer side of the upper arm, chiefly the left arm, gave good reason to suspect a connection with an inoculation *Investigations revealed that all of the patients had been inoculated* subcutaneously in the upper arm with an influenza vaccine In 2 cases there had been an acute local reaction and a short time afterwards an induration in the same place was observed The other patients did not notice an induration until between 2 and 6 months after the inoculation The period between the inoculation and the excision of the nodule varied between 4 and 18 months

In 11 cases it was possible to obtain information about the manufacture and composition of the vaccine 9 patients had been given aluminium-oxide adsorbed vaccine and 2 saline vaccine without any adjuvant. Though this information may not be entirely reliable there is no doubt nevertheless that aluminium-oxide adsorbed vaccine was used in the majority of cases

The histological appearance of the granulomata with large necroses

and, in an early stage, excessive infiltration of eosinophilic leucocytes seems to indicate an allergic origin, for example a reaction to egg albumin in the vaccine. Intradermal skin tests with egg albumin on two patients gave negative results, however. Intradermal skin tests with the vaccine in question also proved negative. Since these investigations did not give any indication as to the origin of the granulomata animal experiments were carried out.

## MATERIAL AND METHODS

The sections were stained by haematoxylin eosin, van Gieson's stain and the Ladewig method.

The following vaccines and preparations were used in the experiments.

contains the virus strains A2/Asian/Formosa, A1/PR301 and B GL1739 54, inactivated with formalin and 0.01 per cent thiomersal.

3. A suspension of commercial aluminium oxide for laboratory use in normal saline with 0.5 per cent phenol.

4. Same as prep. 3 with egg albumin adsorbed on the aluminium-oxide.

5. A suspension of the same γ aluminium oxide as is used in the ASTA vaccine in normal saline with the addition of gelatine and phenol in the same proportions as in the vaccine.

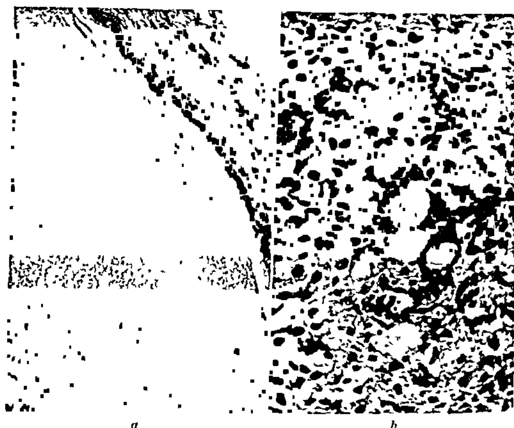
6. Gelatine and phenol in normal saline, as in prep. 5.

7. A suspension of the same γ aluminium oxide as in the ASTA vaccine in normal saline without any other additions.

## RESULTS

A first test series (A) consisted of four groups with three animals in each group. Animals in the two first *groups (A 1 and A 2)* were injected subcutaneously with aluminium oxide adsorbed vaccine (prep. 1). In group A 2 the animals also had an intraperitoneal injection 30 days after the first injection. *In group A 3 the* animals received an injection of egg albumin adsorbed on aluminium oxide (prep. 4) and in group A 4 they had aluminium oxide with phenol (prep. 3). All of the animals were sacrificed after 42 days. Previous intradermal skin tests with the aluminium oxide adsorbed vaccine in groups A 1 and A 2 had given completely negative results.

All six of the animals in the two first groups had peppercornsized yellowish, well defined nodules in the subcutaneous tissue, the fasciae, or subfascially in the musculature at *the site of* injection. Histological examination revealed that the nodules were of a granulomatous character. They consisted of tightly packed, fairly large, oval or elongated cells with light, foamy or *granular* cytoplasm and oval nuclei with



*a* *b*

*Fig 2*

a) Subfascial granuloma in guinea pig 28 days after inoculation of aluminium oxide adsorbed influenza vaccine van Gieson × 60

b) Detail of a) showing macrophages and giant cells with coarsely granular cytoplasm Haematoxylin eosin × 470

scanty chromatin and prominent nucleoli Several mitoses were observed These cells had the character of proliferating macrophages, probably of a fibroblastic origin Occasionally groups of foreign body giant cells were also observed The granulomata contained varying numbers of inflammatory cells, lymphocytes, plasma cells and eosinophilic leucocytes Centrally there were fairly extensive, faintly acidophilic necroses of a finely granular structure These often contained *larger amorphous particles of an unknown nature The granulomata* were surrounded by a thin layer of connective tissue Dense infiltrations of lymphocytes in the boundary zone to the surrounding tissues were not observable as they were in the human cases (Fig 2)

Animals in Groups A 3 and A 4 revealed no granulomata of this type Instead, at the site of injection a foreign, non stainable material was observable presenting very sparse fibroblastic proliferation and occasional foreign body giant cells in the vicinity (Fig 3)

The second test series, B, consisted of five groups each with three animals Group B 1 was injected subcutaneously with aluminium-oxide adsorbed vaccine (prep 1) and the animals were sacrificed 9, 19 and 28

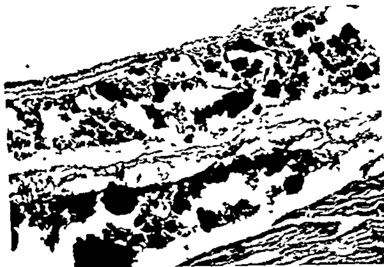


*Fig 3*

Intrafascial lesion in guinea pig 42 days after injection of large grained aluminium oxide for laboratory use Large and compact clumps of foreign material surrounded by a few fibroblasts and single giant cells Haematoxylin eosin × 130

days after the injection All three animals revealed typical granulomata The 9 day old granuloma however, showed much less extensive central necrosis than the older ones

Group B 2 was injected with saline vaccine (prep 2) and sacrificed after 42 days These animals showed no macroscopic lesions Histological examination of the injection area, however revealed in one of the animals a small focus of newly formed fibroblasts and lymphocytes superficially in the musculature The changes were insignificant and uncharacteristic and showed no tendency towards necrosis

Groups B 3 and B 4 were injected with a suspension of γ aluminium oxide (preps 5 and 7) These animals were sacrificed after 42 days and 19 days respectively All of the animals in these two groups revealed typical granulomata Animals in group B 5 received one injection of gelatine and phenol in normal saline (prep 6) and were sacrificed after 42 days None of these animals revealed any changes at the site of injection

## DISCUSSION

The experiments have shown that influenza vaccine containing aluminium oxide injected subcutaneously into guinea pigs consistently results in characteristic granulomata built up of macrophages and with central necrosis Saline vaccine gives no such reaction Similar granulomata likewise results from the injection of a simple suspension of the γ aluminium oxide which is included in the vaccine Thus it is evi

dent that the characteristic tissue reaction is brought about by aluminium-oxide and that there is no question of an allergic reaction to egg albumin or to the virus in the vaccine. If, however, ordinary laboratory aluminium-oxide is injected in the same way no granulomata result and there is only a fairly slight foreign body reaction The γ-aluminium oxide which is used in the vaccine differs only from the laboratory preparation in the sizes of the particles In the preparation of the vaccine the object has been to reduce the size of the particles in order to attain the maximum absorbing surface (5, 7 15)

Fine grained aluminium-oxide injected into the tissues in guinea pigs thus results in a lively proliferation of macrophages forming a granulomatous lesion Through disintegration of the macrophages a central amorphous area appears consisting both of necrotic material and of aluminium-oxide residue The granulomata yield peripherally to increasingly profuse fibrosis

In certain respects the granulomata in man differs from those which were produced in the animal experiments This difference, however, can be explained by the fact that in humans the process was investigated at a later stage when the fibrosis had become much more pronounced and the macrophages for the most part had disappeared Thus the origin is certainly the same, *i e* a reaction to fine grained aluminium-oxide It is true that 2 of the 15 patients possibly had been injected with a saline vaccine, but as already pointed out, this information is not entirely reliable

In one respect, however, there is a notable difference between the human granulomata and those produced in guinea pigs In man the formation of lymphoid tissue at the periphery of the granulomata is highly characteristic This reaction was completely absent in the experimental granulomata irrespective of whether aluminium-oxide adsorbed vaccine or a simple suspension of aluminium-oxide was used

During recent years many reports on mass inoculations against influenza have appeared Acute local reactions are reported to be very common, but these are usually mild and transient (1, 2, 3, 8) On the other hand delayed, persistent local reactions are seldom mentioned Vaccines of the incomplete *Freund* s adjuvans type sometimes give rise to sterile abscesses or paraffinomae (4, 10, 11, 14) The frequency of such reactions seems to have been less than 1 per cent and has been reduced in recent years through the introduction of increasingly pure preparations (17) Oil emulsions with high viscosity have a greater tendency to produce abscesses and foreign body reactions than oil emulsions with low viscosity Sometimes persistent local infiltrates may also occur following the use of vaccines containing aluminium phosphate (9, 10) Himmelweit claims that the inoculation of mice with such vaccines results in a high frequency of sterile abscesses, but on the other hand this is seldom the case in humans (6) The author examined a number of infiltrates which developed locally in children after

the injection of a triple vaccine (tetanus, diphtheria and whooping cough) containing aluminium phosphate These infiltrates revealed a histological picture similar to the influenza vaccine granulomata although there seemed to be a more pronounced tendency towards liquefaction A one-year-old granuloma following the inoculation of aluminium oxide adsorbed influenza vaccine has been described by *Voss & Tolki (18)* This showed a histological structure which seems to have been identical with the one of the present cases Finally, granulomata of a similar nature have been produced in guinea pigs by the injection of influenza vaccine containing aluminium-hydroxide (12)

Concerning the frequency of granulomatous lesions at the site of injections with aluminium-oxide adsorbed influenza vaccines it is difficult to draw any definite conclusions As far as the author knows, there is no information about this in the literature The fact that in guinea pigs injections of these vaccines consistently result in granulomata does not necessarily imply an equally high frequency in man, since *the animals received a much higher dose as compared to their weight* Likewise it is possible that the animals' tissues are more sensitive During 1959 and 1960 aluminium-oxide adsorbed influenza vaccines were used widely in group inoculations in Sweden, when many thousands of people were inoculated Thus the 15 cases dealt with here represent a fraction only of the total number On the other hand some of these patients reported that many who had been inoculated at the same time also had experienced a local development of nodules, but they had not been sufficiently worried to seek medical aid Probably the frequency is not altogether as small as this, but it arouses no great attention on account of the insignificant subjective discomfort

The granulomata in question are of a certain practical interest both to the surgeon and to the pathologist, from the differential-diagnostic point of view since the patient often fails to associate the "tumour" with the inoculation Clinically they cannot be distinguished from genuine tumours since they are hard and indistinctly defined Histologically older granulomata with pronounced fibrosis can be very reminiscent of rheumatic granuloma (Fig 1 b) while the fresher ones may suggest specific inflammation So far as is known there has never been a case of genuine tumour developing at the site of an influenza vaccine inoculation

## SUMMARY

15 subcutaneous lesions appearing at the site of influenza vaccine injections have been investigated histopathologically These lesions led to medical consultation and subsequent excision 4–18 months after the inoculation In the majority of the cases aluminium-oxide adsorbed vaccine had been used Tests on guinea pigs revealed that local granulomatous lesions consistently resulted from injections with such a vac

cine This tissue reaction was brought about by fine-grained γ alumi nium-oxide, while large-grained aluminium-oxide did not result in any significant reaction Occasionally, similar local lesions may be observed following injections of vaccines containing aluminium phosphate To the pathologist the granulomata are of *differential-diagnostic interest* particularly on account of a similarity often manifest between these and rheumatic granulomata

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# TRANSPLANTATION STUDIES WITH CHROMOSOME LABELLED ERYTHROLEUKAEMIA CELLS OF FOWL

*By*

BENGT LAGERLÖF

Received 13 ix 62

It has been demonstrated in an earlier study that erythroleukaemia in the fowl can be induced as transplantation leukaemia by inoculating intravenously chicks with intact leukaemia cells induced by virus in vitro or in vivo (5, 7). It was found that this form of leukaemia begins as a disseminated tumour disease with no histologic signs of a lag phase in contrast to the virus induced form of the disease, which begins as a focal neoplastic proliferation in the bone marrow, and in which the earliest leukaemic changes do not appear for at least 48 hours. In another study (6) it was shown that, with comparable doses with regard to virus, transplantation leukaemia has a considerably more rapid course than the virus induced disease.

However, intact leukaemia cells inoculated into the chick might release newly formed virus and give rise to a continuous virus infection of the new host. As a result, virus induced leukaemic transformation of the host's own bone marrow cells may occur, and the fully developed transplantation leukaemia might thereby be partly superseded by a virus induced component. The transplantability of erythroleukaemia and its capacity for dissemination at different stages of development of the disease may be studied by ascertaining the extent to which the transplanted cells are responsible for the leukaemic proliferation in transplantation leukaemia and the degree to which it consists of a virus induced autochthonous component.

To evaluate the ratio between transplanted and autochthonous cells present in transplantation leukaemia the transplanted cells must be labelled in some way. A convenient method for identifying the donor cells in the new host is based on the fact that the cell of the male chick has 6 pairs of macro-chromosomes, whereas in the female the fifth is unpaired (1, 2, 3, 9). By inoculating the female chick with leukaemia cells from the male and differentially counting the cells of each sex

This work has been supported by a grant from the Swedish Cancer Society.

it is possible to find to what extent the virus-transformed bone marrow cells of the host are involved in the leukaemic infiltration of various organs. The inoculation of the female chicks having 11 macro chromosomes in their tissue cells with male cells having 12 macro chromosomes may result in low values for the counted number of transplanted cells since any accidental loss of chromosomes incurred during the preparation will tend to give an apparent increase in the number of female cells.

This method was applied in the present study. The ratio of transplanted to autochthonous leukaemia cells was determined in the bone marrow, which is the target organ for the virus, and in the spleen which reflects the peripheral tumour infiltration.

## METHODS

The erythroleukaemia virus used in the experiments and the disease produced by it have been described in previous papers (4, 5). Over the 15 years during which the White Leghorn strain used at this laboratory has been applied in experiments on erythroleukaemia it has displayed no major variation in susceptibility to the virus.

Leukaemia was induced in the chicks by intravenous inoculation of 1 ml of a virus preparation consisting of lyophilized medium from cultures of erythroleukaemic bone marrow diluted with the corresponding volume of nonlyophilized medium (4). This treatment resulted in nearly 100 per cent takes of the disease with gross peripheral infiltration of leukaemia in 7-8 days. In the final stage a group of the chicks was treated with colchicin for chromosome analysis (see below and Table 1).

Leukaemic male chicks that were to be the cell donors to the female chicks were inoculated intravenously in the final leukaemic stage with about 0.5 ml of a 0.5 per cent heparine solution (Vitrum). After 5 minutes the chicks were bled to death by incision of the jugular vein. The blood was centrifuged for 15 minutes at 1200 r.p.m. After pipetting off the plasma the buffy coat was washed in 70 ml Ringer's solution and centrifuged for 10 minutes at 1000 r.p.m. The leukaemia cells were then suspended in Ringer's solution and after counting in a Fuchs-Rosenthal chamber they were injected intravenously in doses of $1 \times 10^{7}$ into 8-10 day old chicks. After 3, 4 and 5 days stained smears of peripheral blood were made for differential cell counts and a suitable number of chicks were treated with colchicin as described below. Check of the sex was made at autopsy and the material from the female chicks was treated further for chromosome analysis.

The leukaemia cells were prepared for chromosome analysis in the following manner. After an intraperitoneal injection of 0.1 ml of 10 per cent alcohol to dilate the vessels mitosis was arrested by intraperitoneal injection of 0.04 per cent Colcemide solution (Ciba), the amount used was 0.01 ml times the body weight in grams. Two hours later this procedure was repeated. Most of the chicks were sacrificed 5 hours after the last colchicin treatment but a few of the chicks which were moribund were killed after only 2-4 hours.

The bone marrow in the femur and the upper part of the tibia was aspirated with a syringe and suspended for 9 minutes in 1.12 per cent sodium citrate at 40° C. The suspension was then centrifuged at 4° C at 1200 r.p.m. for 2 minutes through a nylon cloth with a pore size of about 28 microns. Coarse aggregates of cells that would interfere with subsequent squashing were collected in the nylon filter so that the sediment remaining after centrifuging consisted only of single cells and small clusters. The supernatant was pipetted off and the sediment fixed in a mixture of cooled glacial acetic acid - absolute alcohol (1 : 3). After storing for 2 hours at 4° C the fixative was replaced with 45 per cent acetic acid. The preparation was then stored at 4° C. After pipetting off the acetic acid two drops of the sediment on a slide were stained for 3-5 minutes with an equal volume of 2 per cent orcein in 60 per cent acetic acid, after which squashing was carried out in the usual manner. The specimens prepared in this manner were examined under a phase contrast microscope at magnifications of 1000 and 1600. As the cells contained only 12 readily

identifiable macro chromosomes wide spreading was not necessary and the risk of accidental loss of chromosomes was thereby lessened Great care was taken to analyse the metaphases which appeared to be intact

Because of the small size of the micro chromosomes an accurate analysis of their number and structure is impossible by the usual cytologic techniques In the present study therefore only an approximate count was made of them

## RESULTS AND DISCUSSION

The accuracy of the method for distinguishing between male and female leukaemia cells was examined as a separate experiment In this the chromosome constitution was analysed in leukaemia cells from bone marrow of 5 male and 5 female chicks in the final stage of virus induced leukaemia The results are given in Table 1

TABLE 1

*The Chromosome Constitution of Virus Induced Leukaemia Cells*

| Sex | No of cells with the chromosome number | | | | | | No of cells with unpaired fifth chromosome | No of cells with paired fifth chromosome |
|---|---|---|---|---|---|---|---|---|
| | 9 | 10 | 11 | 12 | 13 | 14 | | |
| Male | | | 1 | 49 | | | 1 | 49 |
| | | | | 50 | | | | 50 |
| | | | | 48 | 1 | 1 | | 50 |
| | | | 1 | 48 | 1 | 1 | 1 | 49 |
| | | 1 | | 49 | | | | 50 |
| Female | | 2 | 48 | | | | 50 | |
| | | | 50 | | | | 50 | |
| | 1 | 1 | 48 | | | | 50 | |
| | | | 49 | 1 | | | 50 | |
| | | 2 | 47 | 1 | | | 50 | |

As the table shows the macro chromosome constitution of the leukaemia cells displayed little dispersion about the normal 12 for males and 11 for females a result that is in close agreement with the findings of *Bayreuther & Thorell* (1) The occurrence of paired and unpaired fifth macro chromosomes respectively in the males and females was extremely regular In incomplete cells it was usually the third and fourth chromosomes that were missing The supernumary macro chromosomes were difficult to identify some of them were morphologically similar to the third macro chromosomes but most of them were regarded as abnormally large variants of micro chromosomes Fig 1 shows the normal 11 macro chromosomes of a female leukaemia cell and in Fig 2 is seen a male leukaemia cell containing two supernumary macro-chromosomes in addition to the normal 12

The results of inoculating female chicks with leukaemia cells from the male are presented in Tables 2 and 3 Groups of 4 females were treated for chromosome analyses 3 4 and 5 days after transplantation

it is possible to find to what extent the virus-transformed bone-marrow cells of the host are involved in the leukaemic infiltration of various organs. The inoculation of the female chicks having 11 macro-chromosomes in their tissue cells with male cells having 12 macro-chromosomes may result in low values for the counted number of transplanted cells since any accidental loss of chromosomes incurred during the preparation will tend to give an apparent increase in the number of female cells.

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The bone marrow in the femur and the upper part of the tibia was aspirated with a syringe and suspended for 9 minutes in 1.12 per cent sodium citrate at 40° C. The suspension was then centrifuged at 4° C at 1200 r.p.m. for 2 minutes through a nylon cloth with a pore size of about 28 microns. Coarse aggregates of cells that would interfere with subsequent squashing were collected in the nylon filter so that the sediment remaining after centrifuging consisted only of single cells and small clusters. The supernatant was pipetted off and the sediment fixed in a mixture of cooled glacial acetic acid – absolute alcohol (1 : 3). After storing for 2 hours at 4° C the fixative was replaced with 45 per cent acetic acid. The preparation was then stored at 4° C. After pipetting off the acetic acid two drops of the sediment on a slide were stained for 3–5 minutes with an equal volume of 2 per cent orcein in 60 per cent acetic acid, after which squashing was carried out in the usual manner. The specimens prepared in this manner were examined under a phase contrast microscope at magnifications of 1000 and 1600. As the cells contained only 12 readily

of the male leukaemia cells The groups are arranged in the table according to the degree of leukaemic infiltration in the peripheral blood As pointed out above, it was impossible to differentiate between leukaemia cells and normal immature erythroid cells in squash preparations from bone marrow, and the values for the female cells in Table 2 therefore include both normal and leukaemia cells The found percentage of female cells is thus probably higher than that accounted for by the autochthonous leukaemic proliferation, and this will have resulted in a relative decrease in the recorded values for the transplanted cells

TABLE 2

*The Chromosome Constitution of Leukaemia Cells and Immature Erythroid Cells in the Bone Marrow of Female Chicks Inoculated with Leukaemia Cells from the Male*

| Days after inoculation of leukaemia cells | Chick no | No of leukaemia cells per 10 000 cells in peripheral blood | No of cells with the chromosome number | | | | | Cells with unpaired fifth chromosome | | Cells with paired fifth chromosome | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 9 | 10 | 11 | 12 | 13 | No | % | No | % |
| 3 | 1 | < 1 | 2 | | 17 | 30 | 1 | 19 | 38 | 31 | 62 |
| | 2 | 3 | | 1 | 13 | 36 | – | 14 | 28 | 36 | 72 |
| | 3 | 4 | | 2 | 8 | 39 | 1 | 10 | 20 | 40 | 80 |
| | 4 | 16 | | 1 | 5 | 44 | | 5 | 10 | 45 | 90 |
| 4 | 5 | 3 | | | 29 | 21 | | 30 | 60 | 20 | 40 |
| | 6 | 10 | | – | 13 | 37 | – | 14 | 28 | 36 | 72 |
| | 7 | 23 | | 1 | 20 | 28 | 1 | 20 | 40 | 30 | 60 |
| | 8 | 34 | | | 9 | 40 | 1 | 10 | 20 | 40 | 80 |
| 5 | 9 | 14 | | 1 | 19 | 31 | | 20 | 40 | 30 | 60 |
| | 10 | 80 | | | 11 | 38 | 1 | 11 | 22 | 39 | 78 |
| | 11 | 335 | | 1 | | 49 | | 1 | 2 | 49 | 98 |
| | 12 | 1 600 | | | 2 | 48 | | 2 | 4 | 48 | 96 |

As is seen in Table 2 there was a fairly wide variation in the percentage of transplanted cells in the bone marrow No 4 of the chicks examined 3 days after inoculation displayed intense proliferation of the transplanted cells in the bone-marrow No 5, which was examined after 4 days had a low frequency of male metaphases in the bone-marrow, which indicated a poor response to transplantation (The preparation of the spleen from this animal was unsuccessful) After 5 days two of the chicks, nos 11 and 12, showed intense proliferation of the transplanted cells in the bone marrow There was pronounced [illegible]

In the spleens the leukaemia cell population was composed chiefly

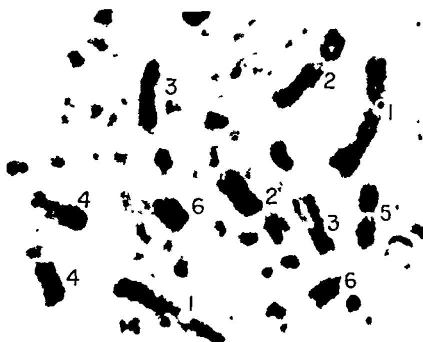



*Fig 1*

Squash preparation of a female erythroleukaemia cell demonstrating the normal composition of 11 macro chromosomes and an unpaired fifth chromosome × 3000

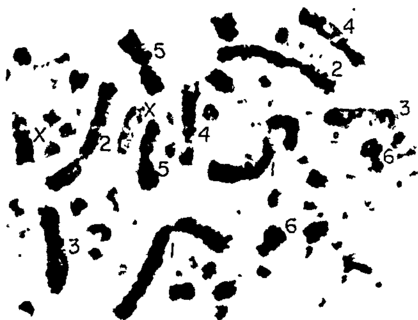



*Fig 2*

Squash preparation of a male erythroleukaemia cell In addition to the 12 normal macro chromosomes there are two supernumary macro chromosomes (indicated by X) × 3000

tive of the concentration of the virus preparation. A possible explanation of this may be that the initial leukaemic transformation in the bone marrow caused by a weak virus preparation is too mild to produce a demonstrably leukaemic blood picture. This is suggested by the fact that, in this form of leukaemia, peripheral leukaemic infiltration does not appear until the disease is far advanced in the bone marrow. Through liberation of virus from existing leukaemic tissue the rest of the marrow might be transformed secondarily, hence the characteristic rapid final stage of the disease. The experiments reported in the present paper may serve as a model for studying the significance of re-infection in the course of virus-induced leukaemia, since the transplanted tumour population might be regarded as the initial leukaemic transformation following inoculation with the virus, and the autochthonous cell population corresponds to cells that have been leukaemically changed by re-infection.

The results indicate that tumourous transformation of the bone marrow in virus-induced leukaemia may be due not only to the initial virus infection but also to re-infection of the bone marrow by virus liberated from primarily transformed cells.

## SUMMARY


The transplantation form of virus-induced erythroleukaemia has been examined by chromosome analysis.

Female chicks were inoculated with leukaemia cells from the male, and an analysis was carried out of the chromosome constitution of cells of bone marrow, which is the target organ for the virus, and of cells of the spleen, which reflect the peripheral infiltration.

The bone marrow displayed a varying degree of proliferation of transplanted leukaemia cells. In the spleen, the leukaemic cell population consisted chiefly of transplanted cells on the third and fourth day after inoculation of transplanted cells, but on the fifth day there was also an autochthonous component.


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of the transplanted cells (Table 3). All but one of the leukaemia cells from the spleens examined 3 and 4 days after transplantation had paired fifth chromosomes. After 5 days the leukaemia cells were still mainly of transplantation origin, but there was now also some infiltration of autochthonous cells. As far as can be judged from the chromosome pattern in the leukaemia cells, there had in these chicks been a leukaemic transformation of their own bone-marrow cells by virus infection, and this autochthonous tumour-cell population had then been superimposed on the transplanted cell population in the peripheral infiltration.

TABLE 3

*The Chromosome Constitution of Leukaemia Cells in the Spleen of Female Chicks Inoculated with Leukaemia Cells from the Male*

| Days after inoc. of 1·10⁶ leukaemia cells | Chick no. | No. of cells with the chromosome number | | | | Cells with unpaired fifth chromosome | | Cells with paired fifth chromosome | |
|---|---|---|---|---|---|---|---|---|---|
| | | 10 | 11 | 12 | 13 | No. | % | No. | % |
| 3 | 1 | | 1 | 24 | | 1 | 4 | 24 | 96 |
| | 2 | 1 | | 24 | | 0 | 0 | 25 | 100 |
| | 3 | | | 25 | | 0 | 0 | 25 | 100 |
| | 4 | | 1 | 49 | | 0 | 0 | 50 | 100 |
| 4 | 5 | | | | | – | | | |
| | 6 | | 2 | 47 | 1 | 0 | 0 | 50 | 100 |
| | 7 | | 1 | 49 | | 0 | 0 | 50 | 100 |
| | 8 | | 2 | 48 | | 0 | 0 | 50 | 100 |
| 5 | 9 | | 4 | 46 | | 4 | 8 | 46 | 92 |
| | 10 | 1 | 2 | 47 | | 3 | 6 | 47 | 94 |
| | 11 | | 1 | 49 | | 0 | 0 | 50 | 100 |
| | 12 | 1 | 1 | 48 | | 1 | 2 | 49 | 98 |

These results bear out those of earlier research by the author in which it was found that transplantation leukaemia has a different mode of origin (5) from, and a more rapid course (6) than, the virus-induced form. The results also confirm the author's view that the virus induced neoplastic character of leukaemia cells can be examined by testing their capacity for producing transplantation leukaemia *in vivo* (7, 8).

It has been shown in an earlier paper that virus infection leads to the formation of multiple small neoplastic foci in the bone-marrow. Leukaemic transformation gradually extends until the bone marrow is eventually pervaded by leukaemic tissue (5). In the previous studies it was also found that inoculation of chicks with virus preparations in increasing dilution delays the onset of the disease, as judged from the presence of leukaemia cells in the peripheral blood (6). In its later stage, on the other hand, the disease follows a rapid course, largely irrespec-

tive of the concentration of the virus preparation A possible explanation of this may be that the initial leukaemic transformation in the bone marrow caused by a weak virus preparation is too mild to produce a demonstrably leukaemic blood picture This is suggested by the fact that, in this form of leukaemia, peripheral leukaemic infiltration does not appear until the disease is far advanced in the bone-marrow Through liberation of virus from existing leukaemic tissue the rest of the marrow might be transformed secondarily, hence the characteristic rapid final stage of the disease The experiments reported in the present paper may serve as a model for studying the significance of re infection in the course of virus induced leukaemia, since the transplanted tumour population might be regarded as the initial leukaemic transformation following inoculation with the virus, and the autochthonous cell population corresponds to cells that have been leukaemically changed by re-infection

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The bone-marrow displayed a varying degree of proliferation of transplanted leukaemia cells In the spleen, the leukaemic cell population consisted chiefly of transplanted cells on the third and fourth day after inoculation of transplanted cells, but on the fifth day there was also an autochthonous component

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kemia 2 [illegible]
Normal [illegible]
m [illegible]
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1 [illegible]
Pa [illegible]
m [illegible] 1960 [illegible] et

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# EOSINOPHILE GRANULOMATOSIS

*Ein ungewöhnlicher Fall mit Veränderungen im Darm, den Lymph-Knoten des Mesenteriums, den Ovarien, im Uterus, der Harnblase und im Herzen*

Von

JOHANNES FOSSGREEN

Eingegangen 26. ix. 62

Das Auftreten von „eosinophilen Granulomen" ist bei einer Reihe von Krankheiten, die sowohl im klinischen und pathologisch-anatomischen Erscheinungsbild, als auch wahrscheinlich in ihrer Ätiologie sehr verschieden sind, beschrieben worden. Beispielsweise genannt seien die eosinophilen Knochengranulome, eosinophile Granulome in der Haut und im Darm, Periarteritis nodosa, Lymphogranulomatosis maligna und Infestation mit gewissen Parasiten. Gemeinsam ist der histopathologische Befund: Granulomatoses Gewebe mit bedeutender eosinophiler Zelleninfiltration. Welche Rolle die eosinophilen Zellen im Krankheitsablauf spielen, ist noch in vielen Punkten ungeklärt. *Weidman* (1947) spricht unter besonderer Bezugnahme auf die eosinophilen Hautgranulome von einem „histologischen eosinophilen Syndrom".

Der folgende Fall erregte Aufmerksamkeit durch zahlreiche, zum Teil monströse und tumorartige „eosinophile Granulome und Infiltrationen".

## KRANKHEITSGESCHICHTE

36-jährige Frau ohne Krankheiten in der Anamnese. Erbliche D[illegible]

[illegible]

6. 4. 2. 5[illegible] [illegible]schmerzen im Epigastrium [illegible]

Patientin [illegible]
chung wa[illegible]
übrigen [illegible]
Dünndarm[illegible]
mutlich [illegible]

[illegible]

TABELLE 1

| Datum | Blutkörperchensenkung mm 1h | Leukozyten | | | | | |
|---|---|---|---|---|---|---|---|
| | | Anzahl | Stabkernige | Segmentkernige | Eosinophile | Lymphozyten | Monozyten |
| 1959 | | | | | | | |
| 7/4 | 17 | | | | | | |
| 13/4 | 24 | | | | | | |
| 20/4 | 25 | | | | | | |
| 23/4 | | 10400 | | | | | |
| 27/4 | | 9600 | 2 | 48 | 19 | 28 | 3 |
| 14/8 | 22 | 13180 | 4 | 62 | 11 | 19 | 4 |
| 26/8 | 35 | 8620 | 2 | 86 | 9 | 12 | 1 |
| 3/9 | 18 | | | | | | |
| 7/9 | 15 | | | | | | |
| 16/9 | 4 | | | | | | |
| 14/12 | 18 | | | | | | |
| 1960 | | | | | | | |
| 14/1 | 4 | 17060 | 2 | 81 | 11 | 6 | |
| 16/1 | 5 | 14220 | | 79 | 8 | 13 | |
| 18/1 | | 15720 | | | | | |
| 19/1 | 3 | 14460 | (Behandlung mit Zytostatica) | | | | |
| 21/1 | | 17720 | | | | | |
| 22/1 | | 26500 | | | | | |
| 23/1 | | 22300 | | | | | |
| 25/1 | 3 | 28800 | | | | | |
| 1/2 | 5 | 17000 | | | | | |
| 8/2 | 4 | 3840 | | | | | |

TABELLE 2

| *Knochenmarkpunktur* | *27.4.1959* | *15.1.1960* |
|---|---|---|
| Hämozytoblasten | 2 | 2 % |
| Promyelozyten | 3 | 7 % |
| Myelozyten | 12 | 9 % |
| Metamyelozyten | 5 | 4 % |
| Stabkernige Neutrophile | 6 | 8 % |
| Segmentierte | 36 | 6 % |
| Unreife Eosinophile | 2 | 16 % |
| Reife | 10 | 5 % |
| Erythroblasten | 6 | 10 % |
| Normoblasten | 5 | 19 % |
| Retikulumzellen | 1 | % |
| Monozyten | 2 | 2 % |
| Lymphozyten | 10 | 10 % |
| Plasmazellen | | 2 % |

phor normal. Urin ohne Befund. Benzidinprobe im Stuhl 3 Wochen lang wechselnd ++ und — danach —. Keine Parasiteneier im Stuhl. Cristapunktur: Knochenmark mit normaler Zellendichte, keine atypischen Zellen oder Granulome (Zellenverteilung: Tabelle 2.). EKG: $P_2$ breit, im übrigen normal. Blutdruck 100/70.

Trotz Diätbehandlung hielten die abdominalen Schmerzanfälle sowie der breiige Stuhl an. Die Temperatur war normal. Einige Tage lang klagte die Patientin über Parästhesien in den linksseitigen Extremitäten. Objektive neurologische Symptome *konnten nicht festgestellt* werden und sind auch im weiteren Verlauf der Krankheit nicht aufgetreten.

Juni 1959 trat Menostasie, begleitet von Hitzewallungen, ein Ausserdem klagte die Patientin uber Funktionsdyspnoe, Oppressionsgefühl und Neigung zu Knochelöde men Diese Symptome progrediierten jedoch auch späterhin nicht Die Patientin klagte nun auch uber Hautjucken Die abdominalen Schmerzen wurden heftiger und

minos und fettig glanzend Rectoscopie Moderate Rötung der Schleimhaut und

Der
geta
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Se P

Der Stuhl enthielt keine pathogenen Darmbakterien, in den Kulturen wuchsen jedoch jetzt einige Kolonien Candida albicans Am 2/11-1959 wurde die Patientin entlassen

Ambulante Kontrolle am 14/12 1959 Die Patientin klagte uber heftiges Hautjucken Der Stuhl war breiig Auf der Haut fanden sich zahlreiche Kratzspuren Im Abdomen konnte ein ca 5 × 6 cm grosser Tumor unter der linken Curvatur und ein ca pigeongrosser Knoten unmittelbar unterhalb des Umbilicus getastet werden

Im Laufe der folgenden Wochen wurde der Stuhl wieder zunehmend flussig Ausserdem klagte die Patientin über starke Mudigkeit und Hinfälligkeit sowie hartnackiges heftiges Hautjucken

Am 13/1 1960 err
abgemagert und ka
der Haut fand mar
etwa nipsnadelgros

Am 15/1
ausgehend
mitblramt
auf das Ab
delte

Rontgenuntersuchung des Thorax ohne Befund Hamoglobin 83 75 %, Erythrozyten 3 91 Mill/mm³ Thrombozytenanzahl normal, doch terminal ein Abfall auf 88000 mm³ (vermutlich auf Grund der Behandlung mit Zytotoxica) Prothrombin 4 % nach K-Vitamineingabe auf 58 % ansteigend Se-Calcium 10 6–7,7 mg %, alk Phosphatase 14,2 Einh Se Kalium 2 7 maq/l, Se Protein 4 8 %, Coombs direkter Test negativ Toxoplasmosereaktion sehr schwach positiv Bluttype B Rh



*Fig. 1*

pos. Alkoholschmerzprovokationstest negativ. Sternalmark: Mittlere Zellendichte. Normoblastäre Erythropoese. Keine atypischen Zellen oder Granulome (Zellenverteilung: Tabelle 2).

Die letzten Wochen der Krankheit waren durch therapieresistentes Hautjucken, periodische Abdominalschmerzen und wässrige Durchfälle gekennzeichnet. Das Gewicht fiel auf 38,2 kg ab. Die Temperatur war normal und ante mortem subnormal.

## PATHOLOGISCH-ANATOMISCHE UNTERSUCHUNGEN

*Operationspräparat:* Ca. 1½ m langes Dünndarmstück mit rötlichem Peritonaeum. Die Darmwandung ist überall etwas verdickt und enthält viele erbsen- bis pflaumengrosse Knoten, die teils nur in der Mucosa liegen, teils alle Schichten der Darmwand einnehmen. In der Mitte des Präparates befindet sich ein ca. faustgrosser, etwas puckeliger Tumor, der die ganze Zirkumferenz des Darmes umschliesst. Die Schleimhaut ist überall von Ulzera verschiedener Grösse durchsetzt. Im Bereich des grossen Tumors ist das Darmlumen auf eine ca. mandaringrosse, unregelmässige, ulzerierte Kavität erweitert. Alle Tumoren sind fest und bestehen aus weiss-gelblichen Gewebe. Im Mesenterium befinden sich mehrere, bis zu wallnussgrosse Lymphknoten mit gelblicher Schnittfläche.

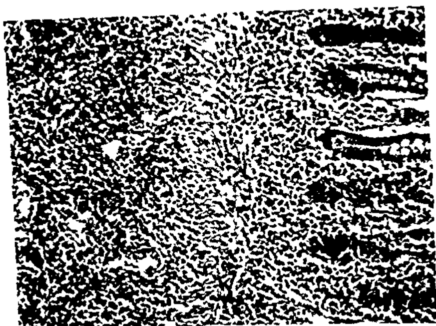


Fig 2

*Histologische Untersuchung* In allen Schnitten finden sich die gleichen Veranderungen doch in variierendem Ausmass Wo die Veranderungen am leichtesten sind liegt zellenreiches Granulationsgewebe bestehend aus Fibroblasten Kapillaren ausserordentlich vielen reifen eosinophilen Granulozyten einigen Lymphozyten und Plasmazellen unter intaktem Darmepithel Der Prozess scheint im lymphatischen Gewebe der Schleimhaut seinen Ausgangspunkt zu haben (Fig 2) In anderen Abschnitten haben sich die Veranderungen augenscheinlich weiterentwickelt und dehnen sich bis in die Submucosa und Muscularis aus (Fig 3) In diesen Bereichen ist die Schleimhaut ulzeriert Die grossen Knoten haben die Darmstruktur vollig zerstort und bestehen aus einem zellenreichen mehr oder weniger vaskularisierten fibrosen Gewebe welches von einigen Lymphozyten Plasmazellen und einer sehr grossen Anzahl eosinophiler Granulozyten durchsetzt ist Die Blutgefasse sind normal strukturiert In ihrem Lumen befinden sich zahlreiche eosinophile Granulozyten Reed Sternbergsche oder Langhanssche Riesenzellen sind nicht gefunden worden

In den stark vergrosserten Lymphknoten sind die Strukturen im Grossen und Ganzen erhalten Die Keimzentren sind etwas vergrossert und in den zentralen Abschnitten befinden sich recht grosse helle kantige Zellen epitheloiden Aussehens Stellenweise Anhaufung von eosinophilen Granulozyten In einem der Lymphknoten befinden sich *kleine* Nekrosen die teilweise von grossen Retikulumzellen und einzelnen un



Fig 3

charakteristischen Riesenzellen des Fremdkörperchentyps umgeben sind (Fig 4) Es befindet sich hier ausserdem eine bedeutende Anzahl eosinophiler Zellen

Es handelt sich um einen eigenartigen, proliferativen und exudativen Prozess von granulomatösen bzw tuberculoidem Aufbau, mit ausserordentlich starker Infiltration von eosinophilen Granulozyten Die Veränderungen entsprechen nicht dem Bild, das man gewöhnlicherweise bei Lymphogranulomatosis maligna oder anderen Formen von malignen Retikulosen findet Eine besondere Variante der Lymphgranulomatosis maligna lässt sich jedoch nicht mit voller Sicherheit ausschliessen Es besteht ausserdem Verdacht auf einen allergisch bedingten Prozess (Carl Jakobsen)

In der Biopsie eines Darmtumors befinden sich 2 eigentümliche, *runde, ca 35 μ grosse Körperchen eingelagert in das eosinophile Granulationsgewebe* Sie enthalten zahlreiche, dünne, bläuliche Körner und einige etwas grössere, helle, stäbchenartige Formation, samt eine zentrale eosinophile Struktur (H-E Präparat) Die Körperchen konnten Parasiten ähneln (Fig 5) Ihre Natur hat sich jedoch nicht aufklären lassen (J Vesterdal Jørgensen) In allen übrigen Präparaten des Operations-und Sektionsmaterials hat man keine dieser Körperchen finden können

*Sektionsbeschreibung* Die Haut ist überall bräunlich Die periferen Lymphknoten sind nicht geschwollen In der Bauchhöhle befinden sich ca ½ l gelblich, klare Flüssigkeit Die Darmwandungen sind fast überall von bis zu hühnereigrossen, mit Peritonaeum bekleideten Tumoren, gleichen Aufbaus wie im Operationspräparat, durchsetzt Die Leber ist, abgesehen von leichter Muskatnusszeichnung, normal In der Porta

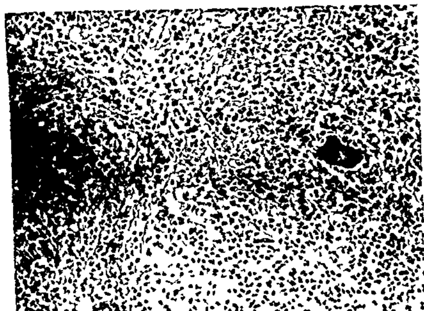


Fig 4

hepatis und im Mesenterium befinden sich mehrere bis zu wallnuss grosse feste Lymphknoten mit gelblicher Schnittflache Die Milz ist klein ohne Infiltration und von fleischiger Konsistens Der Uterus ist knapp huhnereigross Die Ruckwand ist verdickt und von einem festen gelblichen Gewebe infiltriert Beide Ovarien sind zu ca apfelsingrossen glatten sehr festen Tumoren umgebildet und liegen in der Fossa Douglasi Sie bestehen aus graugelblichen Gewebemassen Nieren und Ure

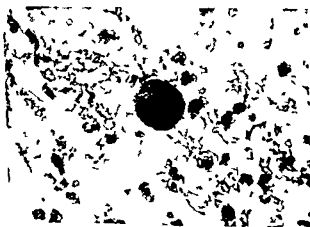


Fig 5

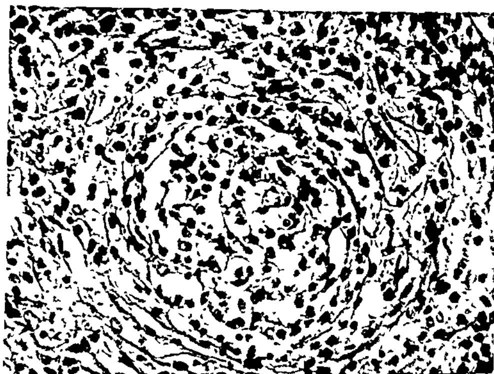


*Fig 6*

teres ohne Befund Der Harnblasenfundus ist verdickt und von graugelblichen Gewebe infiltriert Das Herz ist klein (8 × 6 cm) In der Wand der rechten Vorkammer befindet sich ein ca kirschkerngrosser Knoten mit dunkelroter Schnittflache Im ubrigen ist das Herz ohne Befund *Die Lungen sind, abgesehen von leichtem Emphysem, normal* Wirbelmark normales Aussehen Gehirn ohne Befund

*Histologische Untersuchung* Darm und Lymphknoten (Porta hepatis) Die Veranderungen entsprechen der obigen Beschreibung *Ovartumoren Fibroses Bindegewebe mit bedeutender Infiltration* eosinophiler Granulozyten Stellenweise scheinen die Veranderungen von kleinen, konzentrischen, vermutlich aus Reticulumzellen bestehenden Granulomen auszugehen (Fig 6) Normales Ovargewebe ist nicht vorhanden Salpinx normal Die Ruckwand des Uterus ist von Fibroblasten und eosinophilen Granulozyten infiltriert Am starksten sind die Veranderungen in den periferen Schichten des Myometriums Stellenweise sind die Gefasse ganz oder partiell thrombosiert Das Endometrium ist atrophisch Im Parametrium befinden sich einzelne mikroskopische Granulome gleichen Aufbaus wie oben beschrieben Vagina normal Harnblase Die Muscularis ist von fibrosen Bindegewebe und zahlreichen eosinophilen Zellen infiltriert Die subperitonaeale Schicht ist verdickt und von einigen kleinen Nekrosen durchsetzt Die Schleimhaut ist intakt Rechte Vorkammer. *Der makroskopisch beschriebene Knoten* besteht aus granulomatosem Gewebe mit vielen eosinophilen Granulozyten Vereinzelt kleine Nekrosen, in einer von ihnen liegt eine ne-

krotische Arteriole Knochenmark (Wirbel) Maximal hyperplastisch mit bedeutender diffuser eosinophiler Zellenvermehrung keine Granulome Milz Normal abgesehen von leichter Fibrose und Hamosiderose Leber Nieren und Nebennieren normal Ein Hautpraparat liegt nicht vor

Bakterien Fungi saurefeste Stabchen oder bekannte Parasiten bzw Parasiteneier sind nicht gefunden worden Die Zellen der Granulome enthielten keine sudanophile Substanz und liessen sich nicht mit van Giesons Bindegewebefarbung anfarben

## DISKUSSION

Eine atiologische Erklarung hat man nicht finden konnen Eine Infektion mit Bakterien oder Pilzen hat sich nicht verifizieren lassen Kurz vor dem Tode wurde eine schwach positive Toxoplasmosereaktion gefunden diese muss jedoch zweifellos als unspezifisch gewertet werden In den Faeceskulturen wuchsen einige Kolonien Candida albicans Diesem Befund kann jedoch kaum Bedeutung beigemessen werden da Candida albicans normalerweise bei einer grossen Anzahl Menschen in der Darmflora vorkommt (*Simons* 1953) In einem der Darmtumoren befanden sich zwei eigentumliche parasitahnliche Korperchen die aber nicht naher identifiziert werden konnten Da es sich offenbar um einen Einzelfund handelt ist es unwahrscheinlich dass die Korperchen eine pathogenetische Rolle gespielt haben Die von Parasiten hervorgerufenen eosinophilen Granulome sind auch bedeutend kleineren Ausmasses (*Mercer et al* 1950 *Faust* 1955 *Kruipers et al* 1960) Eine Cryptococcose (*Littman & Zimmerman* 1956) und Coccidioidomycose (*Baum & Schwarz* 1955) meint man ebenfalls ausschliessen zu konnen Eine bekannte Allergie hat nicht vorgelegen

Klinisch wurde der Fall als Lymphogranulomatosis maligna angesehen

Lymphogranulomatosis maligna kommt nur selten primar im Darm vor (*Jackson & Parker* 1947 *Cohen & Canter* 1959) Das klinische Bild ist nicht typisch Die Krankheit kann mit allgemeinen Symptomen wie Fieber Anorexie ... rung ... beginnen ... stand *Bjerkelund* ...

Vergrosserung de... Lymphknoten tritt nicht auf In einigen Fallen ist Hautausschlag beobachtet worden Pruritus wird nicht erwahnt Das Blutbild ist uncharakteristisch Der Alkoholschmerzprovokationstest fallt negativ aus (*Cohen & Canter* 1959) Pathologisch anatomisch unterscheidet man zwischen der ulzerativen infiltrativen und polyposen Form Die beiden letztgenannten sind jedoch auch oft ulzeriert (*Ralston & Wasdahl* 1958) Die Prozesse ... finden sich in der ... ionaren Lymph... findet man das

bekannte Bild mit Reed-Sternbergschen Zellen *Cohen & Canter* (1959) heben doch hervor, dass die Diagnose auf Grund der fast immer gleichzeitig vorkommenden Entzündungsreaktion erschwert werden kann

In unserem Fall fanden sich jedoch auch schwere Veränderungen in den Geschlechtsorganen, der Urinblase und im Herzen Der klinische Verlauf lässt vermuten, dass die Ovarien frühzeitig angegriffen gewesen sind, da unregelmässige und schwache Blutungen mit zu den ersten Symptomen gehörten

Aus *Jackson & Parker*'s (1947) Material, welches 95 Fälle mit Hodgkin's Granulom und Sarcom umfasst, geht hervor, dass Lymphogranulomatosis maligna sowohl primär, als auch sekundär ausserordentlich selten in den Geschlechtsorganen und der Urinblase auftritt Das Myocardium war in keinem der Fälle angegriffen

In unserem Fall fand man eine symptomatische Sprue Diese tritt auch bei anderen Krankheiten, wo eine Blockierung der Lymphgefässe des Darms oder Mesenteriums und/oder eine Zerstörung der Darmschleimhaut vorliegt, auf (*Bjerkelund 1950, Sleisenger et al* 1935) Hautjucken ist ebenfalls kein für Lymphogranulomatosis maligna spezifisches Symptom Auffallend erscheint es, dass die Temperatur und Blutkörperchensenkung im Wesentlichen normal waren und dass die Leber, Milz und Lungen, trotz der bedeutenden Ausbreitung der Prozesse, nicht mit angegriffen waren Das histologische Bild ist nicht typisch für Lymphogranulomatosis maligna Man kann jedoch nicht mit voller Sicherheit die Mögichkeit, dass es sich um eine eigenartige Erscheinungsform dieser Krankheit handeln kann, ausschliessen

Auf Grund des histologischen Befundes und der fehlenden Knochenaffektion, sieht man es als äusserst unwahrscheinlich an, dass eine Histiozytosis X (*Lichenstein* 1953) vorliegen könnte Eine Eosinophilie leucaemoides (*Engbak et al* 1942) meint man ausschliessen zu können und ebenfalls die Krankheiten, bei denen eosinophile Granulome zusammen mit einer Vaseulitis auftreten wie z B Periarteritis nodosa, allergisch granulomatöse Angiitis (*Churg & Strauss* 1951), Endocarditis fibroplastica mit Bluteosinophilie (*Löffler* 1936), eosinophile Prostatagranulome (*Richards & Rubin* 1958) und die bei Kindern beschriebene „Disseminated eosinophilic „Collagen Disease" (*Engfeldt & Zetterström* 1956)

Als Krankheitseinheit werden eosinophile Granulome im Tractus digestorius beschrieben Sie treten entweder als diffuse, rohrförmige Verdickungen oder als solitäre Knoten auf (*Ferrer & Davis* 1957 *Koneman et al* 1959) Eine eosinophile Peritonitis kan gleichzeitig auftreten (*Harley et al* 1959) Die Krankheit ist benign und ein Übergreifen auf andre Organe ist nicht beschrieben Unser Fall kann deshalb kaum in diese Gruppe gehören

*Adams & Kraus* (1950) berichten von einem Fall, der in mehreren Punkten dem unserigen gleicht Bei einer 73 jährigen Frau fanden sie eigentümliche, bis zu hühnereigrosse, grau gelbe, stellenweise nekro-

tische, eosinophile Granulome in der Haut, den Lungen und Nieren Die Granulome bestanden aus Fibroblasten, zahlreichen eosinophilen Granulozyten und in den Lungen ausserdem aus Riesenzellen Vereinzelt waren die Gefässe affiziert Die Patientin hatte Lungensymptome, Herzinsuffizienz, juckenden Hautausschlag, moderate Leukozytose und Bluteosinophilie Eine Diagnose konnte nicht gestellt werden

Auch unser Fall hat sich nicht rubrizieren lassen Auf Grund des histologischen Befundes hat man ihm die morphologische Bezeichung „Eosinophile Granulomatosis" gegeben Man vermutet, dass die Eosinophilie als Ausdruck einer Antigen-Antikorper-Reaktion, entweder auf korpereigene Abbauprodukte oder ein nicht erkanntes Agens, angesehen werden muss (*Hjortling* 1938, *Voorhorst* 1959, *Smith* 1939)

## ZUSAMMENFASSUNG

Es wird von einem Fall mit multiplen, teilweise monstrosen, tumorartigen, eosinophilen Granulomen und Infiltrationen im Darm, den Lymphknoten des Mesenteriums, den Ovarien, im Uterus, in der Harnblase und im Herzen berichtet Die Krankheit dauerte 1½ Jahre und war klinisch durch Gewichtsverlust, Menostasie, abdominale Schmerzen, symptomatische Sprue, Hautjucken, Leukozytose und moderate Bluteosinophilie gekennzeichnet

Histologisch bestanden alle Veränderungen aus einem eigenartigen, exudativen und proliferativen, stellenweise nekrotischen, granulomatosen Gewebe mit massiver eosinophiler Granulozyteninfiltration Auf Grund dieses Befundes hat man dem Fall die Bezeichnung „Eosinophile Granulomatosis" gegeben

Die Ätiologie konnte nicht geklärt werden, und eine Rubrizierung unter bekannte Krankheiten war nicht möglich Eine ungewöhnliche Erscheinungsform der Lymphogranulomatosis maligna Hodgkin wird erwogen

Es wird vermutet, dass die bedeutende Gewebseosinophilie als Ausdruck einer Antigen Antikorper-Reaktion zu werten sei

## SUMMARY

An unusual case of multiple, partly monstrous, tumorlike, eosinophilic granulomas and infiltrations in the bowel, mesenterial lymphnodes ovaries, uterus, urine bladder and heart is presented The disease lasted for 1½ year characterized by wasting, menostasia, abdominal pains symptomatic sprue, itching, leucocytosis and moderate eosinophilia of the blood

Histologically an unusual, proliferative and exudative, partly necrotic, granulomatous tissue with massive infiltration of eosinophilic granulocytes was found According to this the case has been named "Eosinophilic Granulomatosis"

The etiology could not be explained and it was impossible to classify the disease further It has been considered if an unusual variety of Hodgkin's disease might be an explanation

The remarkable tissue eosinophilia is supposed to be the expression of an antigen-antibody-reaction

## LITTERATUR


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FROM THE UNIVERSITY OF BERGEN SCHOOL OF MEDICINE THE GADE INSTITUTE DEPARTMENT OF PATHOLOGY BERGEN NORWAY

# ULCER CARCINOMA OF THE STOMACH IN A 10 YEAR-BIOPSY SERIES

## *A Follow-Up Study of 19 Patients*

*By*

SVEIN THUNOLD and PER WETTELAND

Received 31 iii 62

The original intention of the present study was to investigate the prognosis in cases with very small early resected gastric cancers including initial ulcer carcinoma and minimal, intra-epithelial or multicentric cancers with or without erosions. However, the present biopsy series revealed the best examples of early gastric malignancy among the cases of ulcer carcinoma. The study was therefore restricted to these tumours with a special view to the prognosis in relation to the grade of stomach wall infiltration. Moreover, the post operative prognosis of gastric ulcer-carcinoma seems to have attracted considerably less interest than its frequency, even though many of these tumours represent gastric carcinomas removed at a very early stage.

For comparison it also seemed of interest to investigate the late post operative results in ulcer cases without manifest carcinoma but with an irregular marginal intra epithelial metaplasia, as this condition is stated to be of possible precancerous nature (*Bormann* 1926).

In general it may be very difficult to distinguish an ulcerating primary carcinoma of the stomach from one developing secondarily in a peptic ulcer. Peptic digestion of a carcinoma *in situ* may give rise to an erroneous inference (*Mallory* 1940). A possible previous ulcer may, on the other hand, be overgrown and finally concealed by a secondary carcinoma. These possibilities may partly explain the wide variation in the findings regarding the frequency of malignant change in chronic gastric ulcers, as this has been stated by different authors to vary between 1 and 100 per cent. However, most recent investigators presume the frequency to be low, giving figures somewhere between 1 and 5 per cent (*Boyd* 1961, *Brown et al.* 19[illegible]2, *Dahl Iversen* 19[illegible] [illegible]
authors have stated their opi[illegible]
it is generally agreed that the [illegible]
sence of certain well defined [illegible] logical criteria [illegible]

## MATERIALS AND METHODS

During the period 1949–1958 a total of 60 823 biopsy specimens was received for *histological* examination at The Gade Institute including 1 673 partially or totally resected stomachs with 947 cases of gastric carcinoma and 726 cases of non-malignant peptic ulcer.

A routine diagnosis of manifest or possible ulcer carcinoma had been made in 50 cases. These specimens were re-examined, new sections usually being stained with Mucicarmin and Alcian Blue combined with PAS in addition for the differentiation of epithelial versus connective tissue mucin. The diagnosis of ulcer carcinoma was based upon the following criteria given by *Hauser* 1926:

1) Histological picture at base and edge with reactive changes typical of chronic peptic ulcers, and
2) Tumour infiltration at edge of ulcers but not in the base, characteristic of ulcer carcinoma.

Doubtful cases were excluded from the present study and the original material was *thereby reduced to 19 cases of definite ulcer carcinoma and 7 cases with marked* regenerative epithelial metaplasia at the edge of a chronic gastric ulcer.

The ulcer carcinomas were not graded according to atypical changes in the cells but classified in four groups according to the depth of stomach wall infiltration:

I Infiltration of the mucous membrane only
II Infiltration into but not below the submucosa
III Infiltration involving the muscular coat also
IV Infiltration extending into or through the serous layer

Information about the patients was obtained from the clinical case reports, questionaries to the patients, their relatives or doctors, or when necessary also from the official death certificate. Pre- and post-operative information sufficient for the present study was obtained in all of the 26 patients.

## RESULTS

### *Frequency of Carcinoma Developing from Gastric Ulcer*

The 19 cases of ulcer-carcinoma observed in 726 patients with peptic ulcers of the stomach gives a frequency of malignant change in gastric ulcers of 2.5 per cent. The corresponding figure for pronounced marginal regenerative metaplasia of non-malignant nature was calculated to be nearly 1.0 per cent (7 cases).

TABLE 1
*Lesions according to Age and Sex*

| Lesion | No. of cases | Average age (years) at operation (range) | Males | Females | M:F ratio |
|---|---|---|---|---|---|
| Ulcer carcinoma | 19 | 60.7 (28–77) | 12 | 7 | 1.7:1 |
| Ulcer metaplasia | 7 | 49.6 (40–58) | 5 | 2 | 2.5:1 |

### *Frequency According to Age and Sex*

Table 1 shows that the average age at operation was 60.7 years for patients with ulcer carcinoma, and 49.6 years for those with benign gastric ulcers showing marked regenerative metaplasia. Both the

youngest patient (28 years) and the eldest (77 years) belonged to the malignant group The sex ratio of males to females was calculated to be 1 7 1 in cases of ulcer-carcinoma, and 2 5.1 in those with benign ulcer with marked metaplasia

*Symptoms and Signs*

Four of the 19 patients with ulcer-carcinoma had suffered dyspeptic symptoms for less than one year, while 9 had an ulcer history of more than 10 years (Table 2) Two of the 7 patients with ulcer-metaplasia had had dyspeptic symptoms for less than one year, and two for more than 10 years

TABLE 2

*Duration of Ulcer History (Previous to Operation)*

| Dyspeptic symptoms | Ulcer-carcinoma (19 pat ) | Ulcer-metaplasia (7 pat ) |
|---|---|---|
| Less than 6 months | 2 | – |
| 6–12 | 2 | 2 |
| 1 2 years | 1 | 1 |
| 2 4 " | 4 | 2 |
| 4 10 " | 1 | – |
| More than 10 | 9 | 2 |

At the time of operation, anaemia and a high erythrocyte sedimentation rate were unusual signs in the patients with ulcer-carcinoma In 17 cases examined haematologically, only two revealed haemoglobin levels below 75 per cent Among those with ulcer-metaplasia the haemoglobin value was 70 per cent in one case only, the others were normal

The erythrocyte sedimentation rate had been investigated before operation in 14 patients with ulcer-carcinoma It was slightly elevated in 7 cases, but in all below 30 mm per hour Only one case of benign ulcer with metaplasia showed a rate slightly above the other six, which were all within the normal range

Occult blood was present in the faeces in 9 out of the 14 cases of malignant disease that were examined, and in one of six cases of ulcer-metaplasia

Free hydrochloric acid in the gastric juice had been determined in 13 cases of ulcer-carcinoma Achlorhydria was found in one case only, while the other 12 showed values between 10 and 50 ml N/10 alkali per 100 ml gastric fluid the majority above 25 This investigation had been performed in only three cases of peptic ulcer with metaplasia, two of these revealed the presence of free acid, and the third achlorhydria

*Indications for Gastric Resection*

These are given in Table 3 It should be noted that malignant disease was suspected before operation in 12 of the 19 cases Malignancy was

not suspected radiologically in 7 of these 12 cases, but the clinical course aroused suspicion. Malignant disease could not be excluded before operation in one case of ulcer-metaplasia, while the other six gave no clinical suspicion of malignancy.

TABLE 3
*Indications for Gastric Resection*

| Indication | Ulcer-carcinoma (19 pat.) | Ulcer-metaplasia (7 pat.) |
|---|---|---|
| *Gastric tumour* | 3 | – |
| Malignant disease suspected | 9 | 2 |
| No effect of diet, long history | 5 | 3 |
| Retention | 1 | – |
| Haemorrhage | 1 | – |
| Perforation | | 2 |

## *Localization of Lesions*

As shown in Table 4, the carcinomatous ulcers were approximately evenly distributed between the lesser curvature and the pre-pyloric region, and the benign ulcers with metaplasia showed about the same relative distribution. Only one ulcer-carcinoma was found on the greater curvature, no lesions were encountered in the fundic region.

TABLE 4
*Localization of Gastric Lesions*

| Region | | Ulcer-carcinoma | | Ulcer-metaplasia | |
|---|---|---|---|---|---|
| | | No | % | No | % |
| | Pre-pyloric | 8 | 42.1 | 3 | 42.9 |
| Corpus | Lesser curvature | 10 | 52.6 | 4 | 57.1 |
| Corpus | Greater | 1 | 5.3 | 0 | 0.0 |
| | Fundus | 0 | 0.0 | 0 | 0.0 |
| Total | | 19 | 100.0 | 7 | 100.0 |

## *Histological Grading*

The benign ulcers with metaplasia were not graded according to their extent, only those with pronounced metaplastic changes were investigated (Fig. 1). The distribution of the malignant ulcers according to the depth of stomach wall infiltration is given in Table 5. The infiltration was *limited to the mucous membrane in four cases only* (grade I), but did not extend below the submucosa in ten patients (grade II, Fig. 2). Infiltration of the deeper layers was observed in five cases (grade III, Fig. 3, grade IV). Histological investigation revealed spread to the regional lymph nodes in three cases, belonging to groups II, III and IV, respectively.



*Fig 1*

Male 40 years Marked regenerative metaplasia with papillomatous growth and glandular hyperplasia without infiltration at the edge of a chronic prepyloric ulcer Intermittent ulcer history for 25 years malignancy not suspected Alive 5 years after resection slight symptoms of retention — No 2337/56 (H E × 45)

*Post-Operative Prognosis*

In Table 5 the deaths from ulcer-carcinoma are plotted in relation to time and histological grade of tumour infiltration Regarding the three cases belonging to groups II, III and IV with metastases to regional lymph nodes, these patients survived operation by 2 and 3 years and less than 2 months, respectively

Four of the 19 patients died within eight weeks of gastric resection, two from grade IV tumours with metastases 3 and 8 weeks after operation, and two from post-operative complications (anuria, pulmonary abscess) 1 and 6 weeks after resection (grade III and II, respectively) The remaining 15 patients all survived for the first year The four patients with grade I tumours all survived for five years except for one, who died in the fifth year after operation from malignant hypertension with terminal cerebral haemorrhage and without clinical evidence of malign-

[illegible]

[illegible] years (grade [illegible]), three 6 years (I, II, III), one 4 years (II) and one 3 years (I) after operation As 9 of the 19 patients were alive



*Fig 2*

Male 74 years – Ulcer carcinoma grade II (lesser curvature)
Ulcer history for 3–4 years malignancy strongly suspected before operation. Alive without evidence of recurrence or metastases 5¾ years after gastric resection
No 6238/55 (H E × 90)

3 years after resection, the present material shows a 3-year survival rate of 47.4 per cent for ulcer-carcinoma of the stomach (Fig 4)

It is not yet possible to determine exactly the 4- and 5 year survival rates for the total material. It is known that four patients are still alive

TABLE 5

*Deaths from Ulcer carcinoma according to Time and Grade of Tumour Infiltration*

| Time after operation | Deaths (no of operated cases grouped series) | | | | |
|---|---|---|---|---|---|
| | I (4) | II (10) | III (3) | IV (2) | All groups (19) |
| 0–2 months | | 1* | 1* | 2 | 4 |
| 2 m–1 year | | | | | |
| 1–2 years | | 1 | | | 1 |
| 2–3 | | 3 | 2 | | 5 |
| 3–4 | | 1 | | | 1 |
| 4–5 | 1§ | 1 | | | 2 |
| 3 year mortality (per cent) | 0 | 50 | 100 | 100 | 52.6 |

* Deaths from post operative complications
§ Ulcer carcinoma not related to cause of death



*Fig 3*

Male 56 years Ulcer carcinoma grade II (lesser curvature)
Ulcer history for 14 years Malignancy suspected radiologically but not at operation Died from metastases 2½ years after gastric resection No 3635/56 (H F × 70)

5 years after operation, and two other patients are also alive and without evidence of recurrence or metastases more than 3 (I) and 4 (II) years respectively, after gastric resection When these two patients and the one who died in the fifth post-operative year from malignant hypertension are excluded, the 5-year-survival rate is 25 0 per cent (Fig 4) It should be noted, however, that the outlook depends markedly upon the grade of tumour infiltration at the time of resection As will be seen from Table 5, the 3 year survival rate was 100 per cent for patients with grade I tumours, 50 per cent in group II and 0 per cent in cases with grade III and IV carcinomas

The seven patients with pronounced epithelial metaplasia were all alive and without symptoms and signs of malignant disease when the present investigation was terminated one 6 years, three 5 years, one 4 years and two 3 years after operation

## DISCUSSION

The observed frequency of gastric ulcers undergoing malignant change (2 5 per cent) is in agreement with the prevailing opinion that malignant transformation in chronic gastric ulcers is uncommon

As the total circumference of gastric ulcers had not been investigated



*Fig 2*

Male, 74 years  Ulcer-carcinoma grade II (lesser curvature)
Ulcer history for 3 4 years malignancy strongly suspected before operation Alive without evidence of recurrence or metastases 5½ years after gastric resection No 6298/55 (H E × 90)

3 years after resection, the present material shows a 3-year survival rate of 47 4 per cent for ulcer-carcinoma of the stomach (Fig 4)

It is not yet possible to determine exactly the 4- and 5-year survival rates for the total material It is known that four patients are still alive

TABLE 5

*Deaths from Ulcer carcinoma according to Time and Grade of Tumour Infiltration*

| Time after operation | Deaths (no of operated cases grouped series) | | | | |
|---|---|---|---|---|---|
| | I (4) | II (10) | III (3) | IV (2) | All groups (19) |
| 0 2 months | | 1* | 1* | 2 | 4 |
| 2 m 1 year | | | – | | |
| 1 2 years | | 1 | | | 1 |
| 2 3 | – | 3 | 2 | | 5 |
| 3 4 | | 1 | | | 1 |
| 4 5 | 1§ | 1 | | | 2 |
| 3 year mortality (per cent) | 0 | 50 | 100 | 100 | 52 6 |

* Deaths from post operative complications
§ Ulcer carcinoma not related to cause of death

histological diagnosis of a chronic ulcer as the starting point for the gastric carcinoma

The clinical laboratory investigations did not suggest any peculiarities concerning ulcer-carcinoma of the stomach It is well known that free hydrochloric acid may be present in the gastric juice in cases of malignant disease of the stomach (*Marshall*) In the present series, however, normal acidity was found more frequently than is usual in cases of gastric carcinoma, thus indicating a connexion with the ulcer-disease Others have made the same observations in patients with malignant ulcers, but the diagnostic criteria were not identical with those used in the present study (*Ekstrom* 1952)

The results of the pre-operative radiological examination in the present cases are in accordance with those of other investigators Among 65 malignant ulcers (primaries as well as ulcer-carcinomas) a diagnosis of malignancy was made radiologically in one and suspected in 37 cases only (*Marshall*) Repeated x-ray examinations seem to be necessary for a possible early diagnosis of gastric ulcer-carcinoma As malignancy was suspected in only 7 of the 19 resected stomachs at the time of the macroscopic examination, it is obvious that the diagnosis might be extremely difficult to exclude radiologically

At the present time there seems to be no completely reliable clinical, biochemical, or radiological data to distinguish a beginning ulcer carcinoma from a benign ulcer Examinations of gastric cytology had not been carried out on the present material, but in recent years this method has proved most useful in the detection of small tumours and surface carcinomas In 258 cases of verified gastric carcinoma and 264 cases of peptic ulceration studied by *Schade* (1960) a total of 19 diagnostic errors occurred, namely 6 false negatives in the cancer group and 13 false positives in the ulcer group Therefore, gastric cytology yields an overall diagnostic accuracy of 96 4 per cent (*Schade*)

In the present material the malignant ulcers were approximately equally distributed in the pre-pyloric region and along the lesser curvature Our material is rather too limited to afford reliable conclusions, but the regional distribution indicates that ulcers in the pre-pyloric region seem to become malignant more frequently than those situated on the lesser curvature It must be borne in mind that the majority of benign ulcers are located in the last-mentioned area (*Boyd*)

Even though the present cases are numerically restricted it is interesting to compare the late results of gastric resection for ulcer-carcinoma with those for primary tumour *Eker & Efskind* found a 3 year survival rate of approximately 35 per cent and a 5 year rate of 25 9 per cent in 1025 cases of partially or totally resected gastric carcinoma *Majima et al* (1955) reported a 3- and 5-year-survival rate of 35 9 and 22 2 per cent, respectively, among 60 cases of ungraded ulcer-carcinoma Both rates were slightly more unfavourable than those observed by the same authors among 422 cases of primary gastric cancer

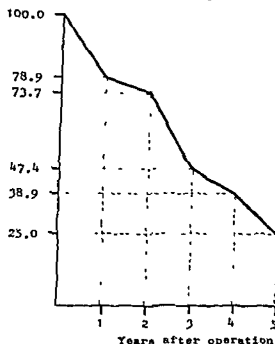



*Fig 4*

X) Observation time only 3 and 4 years for two patients (both without evidence of recurrence or metastases) The 4 year-survival rate is consequently based upon 18 operated cases the 5-year rate upon 16 a third patient also being excluded (died in the fifth year but from a cause unrelated to ulcer carcinoma)

routinely, however, it seems possible that some initial ulcer-carcinomas may have escaped detection In some instances far advanced carcinomas might also have overgrown the original ulcer, and thus made it impossible to determine the sequence of events The observed frequency of ulcer-carcinoma should, therefore, be regarded as a minimum figure

*Gastric ulcers usually occur before the age of fifty*, while most carcinomas of the stomach are diagnosed in patients between fifty and seventy years of age In the present series the transformation of benign to malignant ulcers was chiefly limited to patients of advanced years but was also observed in patients below 30 years of age The calculated sex ratio of 1 7 males to 1 female demonstrates the usual predominance of the male sex generally observed in gastric ulcer and cancer series (*Boyd*, *Brown Fischer & Hazard*, *Eker & Efskind* 1960 *Grimstvedt & Waaler* 1958, *Lahey & Marshall* 1950, *Marshall* 1953)

*Eker & Efskind* and *Lahey & Marshall* found the duration of dyspepsia to be less than one year in 60 70 per cent of patients with gastric cancer In our material a history shorter than one year was recorded only in four cases (21 per cent) 50 per cent of the patients had had ulcer symptoms for more than 10 years, thus supporting clinically the

surface carcinomas, and is advocated as the method of choice for detection of the early cases

For comparison with ulcer-carcinoma, the material also included 7 cases with marked epithelial, regenerative metaplasia at the edge of chronic, benign gastric ulcers The average age in these cases was 49 6 years at operation, and the sex ratio 2 5 males to 1 female Malignancy was suspected previous to operation in one case only All patients were alive and without evidence of malignant disease when the present investigation was terminated 3 6 years after gastric resection had been performed

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In the present study the average 3-year-survival rate amounted to *47 4 per cent, and the corresponding 5-year-rate* was calculated to be 25 0 per cent The slightly more favourable 3-year-outlook observed may indicate a possible slower initial growth of ulcer carcinoma compared with primary tumour However, the average late post-operative prognosis does not seem to be better for cases of malignant ulcers than for those of *primary gastric cancers, but the outlook in a given case* seems to depend largely upon the histological grade of tumour infiltration at the time of gastric resection

The relatively favourable outlook in ulcer-carcinomas removed early should be borne in mind in cases of gastric ulcers which do not heal properly during medical treatment According to *Mallory* it is particularly important that ulcers in the pre-pyloric region should be resected early, and the same conclusion should be drawn from the present study

*It must be mentioned that recent investigators have stated that pati*ents treated with partial gastrectomy for non-malignant ulcers of the stomach show an increased tendency to develop a carcinoma of the gastric stump later on (*Helsingen & Hillestad* 1956) However, this might be due to unknown factors connected with the peptic ulcer disease, and not necessarily to the gastric operation *per se* Anyhow, resection should not be omitted when it is difficult to exclude the presence of a malignant gastric ulcer

## SUMMARY AND CONCLUSIONS

In the present 10 year-biopsy series the observed frequency of gastric ulcers undergoing malignant change was 2 5 per cent (19 of 726 peptic ulcers of the stomach)

The malignant ulcers were predominantly observed in patients past fifty, on an average 60 7 years of age at operation, and the sex ratio was *1 7 males to 1 female*

Malignancy was suspected before operation in 12 of the 19 cases, but radiologically in five only Free hydrochloric acid was found more frequently than is usual in cases of primary gastric cancer

The 19 patients were examined from 3 to 5 years or more after gastric resection The average total 3-year-survival rate amounted to 47 4 per cent The corresponding 5-year-rate could not yet be determined exactly for the whole material Calculated from the available data at present, however, the 5-year-survival rate was found to be 25 0 per cent Thus, the average late post-operative prognosis seemed to be the same as reported in large series of resected primary gastric cancers However, the *prognosis in the present study was decidedly more favourable in* cases where the tumour infiltration had not extended below the submucosa than in those with infiltration of the deeper layers at the time of operation

Gastric cytology has proved most accurate for the diagnosis of small

*D Alessandro & Del Carpio* (1958) employed sodium desoxycholate treatment of Reiter treponemes followed by precipitation with trichloroacetic acid and ethanol They considered that the substance isolated was a lipopolysaccharide which possessed antigenic properties in aqueous solution as the lipopolysaccharide was capable of including the production of antibodies on injection into rabbits in contrast to the polysaccharide prepared earlier which was a haptene However the antigen also reacted with human syphilitic sera in complement fixation reactions (76 out of 85 were positive) This apparent inconsistency with the previously isolated polysaccharides is dealt with further in the discussion

*Pillot et al* (1960) disintegrated Reiter treponemes by ultrasonic vibrations extracted lipid with butanol and ether and precipitated protein at pH 3 5 The polysaccharide remained in solution with a concentration of 1 mg per ml The polysaccharide could also be isolated from the lipid extracted treponemes by digesting the protein fraction with papain Used in a complement fixation test with rabbit anti Reiter serum the polysaccharide reacted in a dilution of 1 320 (serum dilution 1 25) There was no activity with a greater serum dilution (1 200) although an antigen consisting of treponemes disintegrated by ultrasonic vibrations and with a dry weight content of 23 mg per ml was active at this serum dilution No reactivity could be demonstrated with human syphilitic serum either in a complement fixation test or by agar precipitation

*De Bruijn* (1961) showed that the Reiter protein antigen lost its reactivity with syphilitic sera on heating to 100° C for 30 minutes but retained its ability to react with the rabbit anti Reiter sera

By treating the Reiter protein antigen with trypsin for four days and precipitating with ethanol a thermostable substance was isolated which gave a positive anthrone test A substance of polysaccharide nature was thus demonstrable in the Reiter protein antigen This *polysaccharide* was reactive with the rabbit anti Reiter serum also after heating but reactivity of a thermolabile component was found in low dilutions before heating This component was considered to be a *lipid* The Reiter treponeme polysaccharide was non reactive with most of the syphilitic sera examined but reactive with the rabbit *anti Reiter protein* antigen serum This is ascribed to a difference in the polysaccharide fraction between Reiter treponemes and pathogenic *Treponema pallidum* A similar difference could be demonstrated *between Reiter* treponemes and *Treponema zuelzerae* recently cultured from mud by *Veldkamp* (*De Bruijn* 1961)

## MATERIAL AND METHODS

FROM STATENS SERUMINSTITUT, TREPONEMATOSES DEPARTMENT
COPENHAGEN, DENMARK

# STUDIES ON THE ANTIGENIC STRUCTURE OF TREPONEMA PALLIDUM

## *2 Isolation and Purification of Polysaccharides from Reiter's Apathogenic Strain*

*By*

AAGE HEIS CHRISTIANSEN

Received 1 iii 62

In a previous paper (*Christiansen* 1960) on an antigen of polysaccharide nature from *Treponema pallidum* (Nichols), it was suggested that an antigen satisfying the basic requirements of specificity might in fact be found in the treponemal polysaccharide fraction The preliminary experiments were encouraging, but a considerable quantity of material would be necessary to work out a suitable method of isolating the treponemal polysaccharides and preparing them in a pure state As is well known, pathogenic treponemes cannot be cultured *in vitro* and thus the available material is strictly limited It was therefore decided to use the Reiter treponemes as a model for the pathogenic organisms, until the method was fully worked out

The present study deals with the isolation of polysaccharides from the Reiter treponemes and with some of their chemical properties Details of the serological results obtained will be published later

### PREVIOUS STUDIES

*D'Alessandro et al* (1949) treated Reiter treponemes with 2 N NaOH for 24 hours, added trichloroacetic acid until neutralization, and further trichloroacetic acid to a final concentration of 5 per cent The precipitate was removed by centrifugation After adding 4 volumes of ethanol to the supernatant, a precipitate was again produced, which when dissolved in saline (3 mg per ml) and used as antigen in a complement-fixation test, reacted with rabbit anti-Reiter sera With undiluted serum, a strong positive reaction was obtained using an antigen dilution of 1 19,200 The antigen was also able to precipitate antibody, remarkably enough in an even greater dilution (1 300,000) The antigen did not react with human syphilitic sera In view of the thermostable nature of the antigen and its reaction with α-naphthole, the substance isolated was considered to be a polysaccharide

* The study was supported by a grant from P Carl Petersen's Foundation

*Scheme I*
Is lat n of p lysacchar de

*Ly ph l e l Re ter trep nen es*

Ether acetone one hour roo n t mperature

D l j t ze l treponen es → L q t fraction

$H_2O$ then h n xe l for ne l our +4 C

Centr fugat n M S E 000 C n n 4 C

→ H l j l a e (pr te n) d scar led

In l H r s d e

Supernatant $H_2O$ 1

D al z t two days

Ethan l t o l per e nt c ncentrat n

→ S pernatant l car l l

Pr e j tate $H_2O$ 1 Eth a0

M x l w th $H_2O$ f r one hour

Centr fug t on M S E 000 C +4 C

Supernat $H_2O$ → S l ment d car l l

D lysel l o days

Ev perat n n pce 4 C to s x l

Centr f g t n M S E 90 m C 4 C

S pern t $H_2O$ → Scanty s l ment d scar led

Etha l to l per e nt c ncentrat o n

Super ata t ← Se l t $H_2O$ l th a0

Etl an l l 6 per cent c nc

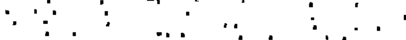
Supe nat nt → Se l nt $H_2O$ 2 Eth 66

Etha l l a per c nt con

S pernatant l a l l Se l nt $H_2O$ l H

*Sej arat n f Nucle c Ac l fr n l lysacch ar le*

As it was imp ssil le t separate the tw sul tances l y fract nal prec pitat n



For the serological investigation sera were used from rabbits immunized with a suspension of lyophilized Reiter treponemes The density of the suspension was twice the international Coli standard 8 Immunization was made by five intravenous injections at intervals of 3 4 days analogous to *D Alessandro s technique* (1953)

The method of extraction is based on the principles quoted by *Morgan & Partridge* (1941) and in particular by *Westphal et al* (1952) When bacteria are treated with a mixture of phenol and water the protein is dissolved in the phenol and the polysaccharide in the aqueous phase The principle of the method has been found applicable to treponemes but it has been necessary to introduce a number of modifications *Extraction and precipitation* unless otherwise stated were done at $+4°$ C and with fluids at this temperature

## PROCEDURE

The lyophilized treponemes are pulverized in an agate mortar and the lipid extracted with a mixture of equal parts ether and acetone approximately 200 ml per g treponemes under magnetic stirring at room temperature for one hour The ether acetone mixture is then decanted and the insoluble residue washed with a little fresh ether acetone mixture which is also decanted The lipid fraction is obtained by evaporation After air drying the insoluble residue at room temperature it is cooled at $+4°$ C and ... water is used per g treponemes ... 22 g of phenol and 6 g of water ... the concentration of phenol becomes about 50 per cent and the suspension is exposed to vigorous mechanical mixing at 6000 r p m for one hour at $+4°$ C in a mixer of the Waring blendor type If the suspension becomes warm mixing is interrupted and the suspension cooled to $+4°$ C It is centrifuged (M S F High Speed 17 Centrifuge with refrigeration) at 20 000 G for 25 minutes whereby the phases are separated into an upper aqueous phase a lower phenol phase and between these a white undissolved layer together with coarser debris at the bottom The aqueous phase $H_2O(1)$ is removed by using a record syringe with a coarse needle so that none of the boundary layer is included This $H_2O(1)$ is dialysed against running tap water until the $FeCl_3$ test for phenol is negative (approximately 48 hours) and then against distilled water approximately 30 l until the $BaCl_2$ test for sulphate and carbonate is negative which takes about six hours if magnetic stirring is used in the dialysis bath If 96 per cent or absolute ethanol is then added to the $H_2O(1)$ to give a concentration of 50 per cent a white precipitate develops which consists of nucleic acid and polysaccharide

After the $H_2O(1)$ is removed by the syringe the needle is inserted through the white boundary layer and as much as possible of the phenol solution is sucked up and discarded Four hundred ml of distilled water is then added to the white layer and the debris in the bottom of the tube this is extracted in the blendor for a full hour with pauses if the temperature rises After extraction the suspension is centrifuged at 20 000 G for 30 minutes The sediment is rather fine and filtration through hard paper may be necessary to remove it completely The supernatant $H_2O(2)$ is dialysed in the same way as $H_2O(1)$ and then concentrated under vacuum at 45° C to about 1/5 of the original volume centrifuged at 20 000 G and a slight amount of undissolved material discarded After addition of ethanol to 50 per cent concentration a precipitate forms which sediments on centrifugation $H_2O(2)$ Eth 50 More ethanol is added to the supernatant from this to 66 per cent and the resulting precipitate $H_2O(2)$ Eth 66 sediments easily by centrifugation Finally ethanol is added to the supernatant from this to 75 per cent whereafter the precipitate $H_2O(2)$ Eth 75 is sedimented by centrifugation After evaporation no more polysaccharide or other antigenic material is found in the supernatant which therefore is discarded

... O(1) Fth 50 $H_2O(2)$ Fth 50 H O(2) ... anol and finally with ether to remove ... they can be transferred to ampoules ... ic acid and polysaccharide while H O

... Fth 66 and ...

... means of ultrasonic ... itative yield of poly... d was discontinued

*Scheme I*
*Isolation of polysaccharide*

*Ly ph l e l Re ter trep nen es*

Ether acetone one hour room t mperature

D I ( l zed tr ponen es → Lq l l fract n

H O phen l t xe l l r one h ur +4 C

Centr f gat on M S E 20 00 0 G r n 3 C

→ I en l ( ha e (pr te n) d scard l

In lul le res due

Supe natant H O I

D l ze l two lays

Ethanol to a l per e nt c ncentrat n

→ Supernatant d car l l

Pre p tat H O I Et ( )

M x l w th H O f r on hour

Centr fugation M S E 20 00 ) C +4 C

Supern t H O → Se l ment l car l l

D al e l two lays

E aporat n in vac 4 C to s v l

C ntr fugat n M S E ) C 4 C

S pernat H O → Scanty se l m nt d scar led

Eth n l to t per e t c ncentrat on

Super atant ← Se l nt H O ) Et 50

Etl an l t 6 per cent c nc

Supernatant → Se l ent H O ? Eth 6?

Etl an l to per cent co c

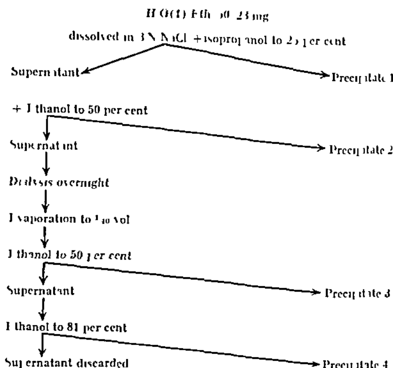
Supernatant d scar l l → Sed nt H O ? Et

*S parat n f Nucle c Ac l fr m l lysaccharide*

As it was imp l le t separate the tw sul tances l y fracti nal precip tati n with ethanol they are presumal ly either bound t gether in a c mplex form or have appr ximately the same s lul il ty rati *Alberty & Heidelberger (1948)* used fractio nati n with is pr panol in the c l l t separate pneum coccal polysaccharide from nucleic acid and the meth d was al f und applicable here An amount of 23 mg

*Scheme 2*

Separation of nucleic acid from polysaccharide




of the $H_2O(1)$ Eth 50 precipitate was dissolved in 45 ml of 3 N NaCl and isopropanol added to 25 per cent at +4° C This resulted in a precipitation of most of the nucleic acid *The remainder of the nucleic acid was precipitated by adding ethanol* to 50 per cent The polysaccharide was not precipitated After dialysis to remove NaCl and concentration *in vacuo* the addition of ethanol to give 50 per cent precipitated a slight
acid
ethan

*Analytical Methods*

Polysaccharide was demonstrated by the ant ... f r *hexoses Two ml of the reagent a 0 2 per cent* $H_2SO_4$ *is mixed with 1 ml of the sample After* trophotometer (Zeiss or Beckmann) at 625 mμ

Demonstration of protein was carried out by Lowry s method (*Lowry et al* 1951, s which contain tyrosine and tryp phenol reagent in the presence of in a spectrophotometer at 750 mμ mple (dry matter) per ml of water t 260 mμ Determination of type of nucleic acid ... nol test for pentoses according to *Dische* (1937) *and DNA by the diphenylamine reaction for desoxyribose (Dische* 1930)

*Serological Methods*

The isolated fractions were examined in the complement fixation test using the technique described previously (*Christiansen* 1960) with 18 hours complement fixation in the cold One mg of antigen was dissolved in 1 ml of water and the antigen dilutions prepared from these stock solutions

## RESULTS

With the method of extraction used, there is a quantitative yield of polysaccharide in the aqueous phases $H_2O(1)$, and $H_2O(2)$, while the protein is found partly in the phenol phase and partly in the undissolved boundary layer (Table 1). The analyses were performed after the phenol had been removed by dialysis.

TABLE 1

*Qualitative Examination of the two Aqueous Phases and the Undissolved Residue*

| Test | Sample: $H_2O(1)$ | Sample: $H_2O(2)$ | Sample: Undissolved residue | Conclusion |
|---|---|---|---|---|
| $FeCl_3$ | — | — | — | No phenol present |
| Folin Ciocalteux | | — | + | Protein present in residue |
| Anthrone | + | + | — | All the polysaccharide in the aqueous phases |

TABLE 2

*Survey of the Coarser Chemical Composition of the Samples Containing Polysaccharide*

| Fraction | Yield as per cent of dry weight | Polysaccharide | Protein | RNA | DNA | Residue |
|---|---|---|---|---|---|---|
| $H_2O(1)$ Eth 50 | 3.0 | 14 % | 14 % | + | — | 72 % |
| $H_2O(2)$ Eth 50 | 1.3 | 57 % | 9 % | + | — | 34 % |
| $H_2O(2)$ Eth 66 | 1.2 | 37 % | traces | — | — | 63 % |
| $H_2O(2)$ Eth 75 | 0.7 | present* | traces | — | — | |

* The yield was too small for quantitative determination

TABLE 3

*Survey of the Coarser Chemical Composition of the Samples from $H_2O(1)$ after Treatment with Isopropyl Alcohol*

| Precipitate | Yield mg | Yield % | Polysaccharide | Protein | RNA | DNA |
|---|---|---|---|---|---|---|
| 1 | 15.5 | 69.2 | (+) | — | + | — |
| 2 | 2.3 | 10.3 | (+) | — | + | — |
| 3 | 2.6 | 11.6 | — | + | — | — |
| 4 | 2.0 | 9.9 | ± | — | | — |

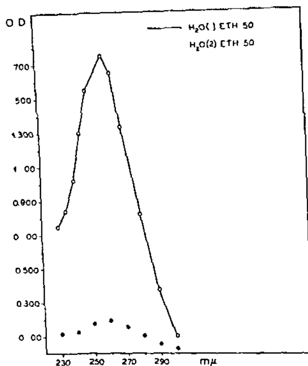
Table 2 shows the yield and composition of the fractions. The percentages are determined on the basis of dry weight, the polysaccharide is expressed by the hexose content, determined by a quantitative anthrone test, and the protein by a quantitative Folin-Ciocalteux test. The nucleic

acid content is demonstrated by a typical absorption maximum at 260 mμ (see Fig 1), and the type of nucleic acid is determined by the methods mentioned above $H_2O(2)$ Eth 75 was present in too small amounts for analysis, but serological studies suggested that the reacting component was identical with the component demonstrated in $H_2O(2)$ Eth 66, although this allowed a greater dilution, suggesting a greater content of antigenic material. The greatest content of polysaccharide is found in $H_2O(2)$ Eth 50, which is also the most reactive of the three fractions in the complement-fixation test (Fig 2) In addition, it is seen that protein can be demonstrated after isolation of the fractions, although in rather small amounts Fig 1 shows the content of nucleic acid in $H_2O(1)$ Eth 50 and $H_2O(2)$ Eth 50 By far the larger amount of nucleic acid is seen to be present in $H_2O(1)$ On treatment with isopropanol, as described previously, $H_2O(1)$ Eth 50 could be fractionated into nucleic acid, protein and polysaccharide (Scheme 2) The results are seen in Table 3, and the proportions of the constituents are of the same order as the analytic results from Table 2 Traces of polysaccharide are found in the nucleic acid fractions, but most by far in precipitate 4, namely 2 0 mg No correction was made for content of pentose, which is known to be present in the form of ribose from RNA, and the somewhat higher value for polysaccharide (14 per cent) in Table 2 may be due to this, as *Johanson* (1953) has shown that in the anthrone test a mixture of arabinose and glucose gives values higher than the equivalent of the glucose content Table 3 also shows that precipitate 1 constitutes 69 2 per cent Comparing this with the figure for the remainder noted for $H_2O(1)$ in Table 2, 72 per cent, it seems probable that this remainder consists mainly or nucleic acid The largest of the fractions, $H_2O(1)$ Eth 50, was found to contain the least polysaccharide, and was completely inactive serologically

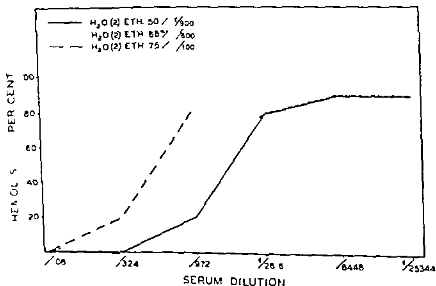
The 2 mg polysaccharide from precipitate 4 was dissolved in 2 ml of water and used as antigen in a complement-fixation test a rabbit anti Reiter serum, but was unable to react

Fig 2 shows the results of a complement fixation test with the three fractions from $H_2O(2)$ and an anti-Reiter treponeme serum The greatest activity is seen to be obtained with $H_2O(2)$ Eth 50, while $H_2O(2)$ Eth 75 is least reactive The antigen dilutions used are chosen on the basis of preliminary experiments to determine the titre of the antigen, i e the maximum dilution giving an optimum haemolysis curve with a given serum

In contrast to $H_2O(1)$ Eth 50, the three fractions from $H_2O(2)$ were found to be sparingly soluble in water at approximately pH 7, especially $H_2O(2)$ Eth 75 The latter, however, could be brought into solution by adding acid to approximately pH 2, although this procedure was not applied further, as the complement-fixation test occurs around the neutral point Solutions of one per cent however could be made by adding a drop of water at a time while mixing The lipopolysaccharides




*F g 1*
Measurement of nucle c ac d content




*F g 2*
Rea t i y f the th r e samples f p l sa charide from $H_2O$(2)

isolated by *Westphal et al* from bacteria of the Salmonella group (*Westphal* 1961) also showed the same solubility characteristics

The lipid content of the Reiter treponemes was found to be approximately 16 per cent, the same order of magnitude as the value of 16.6 per cent found by *Heymann & Siefert* (1958)

## DISCUSSION

The principles described by *Westphal et al* (1952) for the isolation of polysaccharides, were applicable to studies on the treponemes, but with technical modifications The polysaccharide is more firmly attached to the treponemes than to the bactaeria of the Salmonella group In the latter, an extraction time of 5–30 minutes was sufficient, while the treponemes require one hour with phenol-water together with one hour with excess water The latter procedure is a condition for achieving a serologically reactive material, and thus represents a further difference from the usual technique A preliminary extraction of lipid by ether and acetone facilitates the subsequent extraction of polysaccharide The very considerable unanalyzed residues (Table 2) from $H_2O(2)$ Eth 66, together with the solubility conditions and the method of extraction, may signify that the substances isolated are not pure polysaccharides but lipopolysaccharides, as such complexes do not yield the lipid fraction on ether-acetone extraction, but only after acid hydrolysis The results of *Fromme et al* (1958) support this hypothesis They find 2 3 per cent lipopolysaccharides in *Salmonella abortus equi,* the lipid fraction (lipid A) amounting to 24 per cent, while the most interesting fraction in the Reiter treponeme, $H_2O(2)$ Eth 50, amounts to 1 3 per cent and has an unanalyzed residue of 34 per cent The molecular weight has not been determined so far, but is at any rate over 30,000, and is possibly of the same order of magnitude as nucleic acid ($\sim 10^6$), as in ethanol precipitation these substances follow each other closely If $H_2O(2)$ Eth 50 is a high molecular lipopolysaccharide, it should be possible to immunize rabbits with it Experiments have been started to verify this hypothesis

So far it has not been decided whether the nucleic acid content of $H_2O(2)$ Eth 50 plays a part in the antigenic properties of this fraction The probality of this is slight, partly because the RNA in $H_2O(1)$ is serologically inactive, partly because the RNA in $H_2O(2)$ is minimal in relation to the polysaccharide content

Experiments have been started using agar precipitation to decide whether the polysaccharide in the three fractions from $H_2O(2)$ reacts with the same antibody Preliminary observations suggest that this is the case

On the basis of its content of dry substance, $H_2O(2)$ Eth 50 has been found more sensitive than the polysaccharides studied by *Pillot & De Bruijn*, but it did not react in dilutions as high as those used by *D'Alessandro* in 1949, just as the concentration of $H_2O(2)$ Eth 50 had to be increased several hundred times to reach precipitation, while *D'Ales-*

*sandro's* polysaccharide could be diluted further in precipitation experiments.

An examination of 75 syphilitic and non-syphilitic human sera in complement fixation tests, and a similar number of sera from rabbits, both normal and with experimental syphilis, gave wholly negative results. Positive results were obtained only with rabbit anti-Reiter sera. As anticipated, therefore, the isolated polysaccharides must possess pronounced specificity.

The lipopolysaccharides isolated from Reiter treponemes by *D'Alessandro & Del Carpio* in 1958, which reacted with human syphilitic sera, presumably contained impurities, possibly protein. This assumption is shared by *De Bruijn* (1961).


SUMMARY

Using a modification of *Westphal's* phenol water method, polysaccharide could be isolated from Reiter treponemes. This polysaccharide reacts only with rabbit anti-Reiter serum. The Reiter treponeme is shown to contain approximately three per cent polysaccharide, corresponding to the figure obtained in experiments with bacteria of the Salmonella group. A lipid content of approximately 16 per cent is found, in agreement with the results of other authors.

The method of extraction is described, and the results obtained discussed in relation to those of other authors. The hypothesis is put forward that the material isolated is lipopolysaccharide of high molecular weight, and having antigenic properties.

New experiments have been started to verify this hypothesis.


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*D'Alessandro* [illegible] the protein antigen of the [illegible]

*D'Alessandro* [illegible] of the treponemes. Nature [illegible]

*De Bruijn J. H.* Het antigeen in de serologische diagnostiek van syfilis. Thesis. Utrecht 1961.

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saline containing 0 25 per cent phenol The density of the suspension corresponded to twice that of International Coli Standard 8 The content of dry matter was 2 4 mg per ml The injections were given intravenously every 3rd day, in doses of 0 5 1 0 1 0 2 0 2 0 and 2 0 ml respectively, and 3 to 4 days after the last injection blood samples were drawn either by heart puncture or from ear veins One rabbit immune serum Palermo 752, was kindly supplied by Professor G D Alessandro Palermo The following sera were tested in addition to immune sera from rabbits a number of arbitrarily selected reagin-positive and reagin negative human sera sera from 51 verified cases of human syphilis, and a few syphilitic sera from experimentally infected rabbits

*Serological Technique*

Before the serum was examined with the polysaccharide antigen it was tested in one or more of the following reactions

1 Wassermann-Mørch's complement fixation test with cardiolipin antigen C WR-M (13, 14, 15)
2 Kahn's standard test with 'crude' antigen KR (7)
3 Meinicke's clarification test with 'crude' antigen MR (9)

These tests are designated in the following as STS (standard tests for syphilis) Some of the human sera were also examined by the

4 TPI test (10) performed according to the technique described by Nielsen (11)

*Methods of Recording the Results and Definitions of Expressions Used*

The results of complement fixation test with polysaccharide antigen are indicated as percentage haemolysis, thus no haemolysis indicates a maximum positive reaction The results from C WR-M and KR are expressed quantitatively in degrees of strength (8 13)

*Serum titre* The reciprocal value of the highest serum dilution giving 60 per cent haemolysis

*Antigen titre* The reciprocal value of the highest antigen dilution giving 60 per cent haemolysis with a positive control serum under such experimental conditions that the haemolysis curve begins at 0 per cent and finishes at 90 100 per cent (typical curve)

One haemolytic dose of complement is the smallest amount of complement dilution (1 33) required for complete haemolysis of 0 2 ml of a 2 5 per cent suspension of sheep erythrocytes in saline sensitized with 3 haemolytic units of haemolysin

One haemolytic unit of haemolysin is the smallest amount of haemolysin which produces complete haemolysis of 0 2 ml of a 2 5 per cent suspension of sheep erythrocytes in the presence of excess complement

*Technique in Complement Fixation Tests Using Polysaccharide Antigen*

The serum was titrated in saline in 3 fold dilutions and examined by the complement fixation test The tube with the highest serum concentration contained 0 025 ml of serum corresponding to a serum dilution of 1 12 after the addition of antigen and complement The serum dilution in the next tube was 1 36 and so on The antigen dilutions were prepared from a stock solution containing 1 mg of dry matter per ml distilled water A convenient amount of stock solution was diluted with saline to this one volume of saline and one volume of buffered saline at pH 7 38 were added the next day On the basis of preliminary experiments in order to determine the complement titre (one haemolytic dose) an adequate amount of complement was added to the antigen solution and 0 3 ml of the mixture antigen + complement added to the serum dilutions The mixture was then incubated at +4° C for two hours subsequently at 37° C in a water bath for 30 minutes and the haemolytic system then added After one hour at 37° C in the water bath the solution was incubated at +4° C overnight and the haemolysis percentages were read the following day The result is given in haemolysis per cent

In preliminary experiments serum antigen and complement were incubated for 18 hours at +4° C in analogy with the experience gained previously using the polysaccharide antigen obtained from pathogenic treponemes (1) Later however the fixation time was reduced to 2 hours at +4° C plus 30 minutes at 37° C Although this reduced the sensitivity by one dilution step nevertheless it remained satisfactory for the purpose with the sera used

## RESULTS

The preliminary experiments with the polysaccharides Eth 50, 66 and 75 were carried out with a known serum, Palermo 752 This serum was examined in Palermo by the Reiter protein complement fixation test (4) The results are shown in Table 1 The titre for this serum, examined by the RPCF test, ranges between 320 and 640

TABLE 1

*Control Serum Palermo 752 Examined with RPCF by D Alessandro*

| Serum dilution | 1 4 | 1 80 | 1 160 | 1 320 | 1 640 | 1 1280 | |
|---|---|---|---|---|---|---|---|
| RCF | 0 | 0 | 0 | 0 | 80 | 100 | Kolmer technique 1/5 vol |

* Per cent haemolysis

Table 2 shows the determination of complement titre in the presence of antigen The experiment was carried out with an antigen solution containing 0 005 mg of dry antigen per ml, and complement diluted 1 33 in this antigen solution Decreasing amounts of antigen-complement mixture were added to 8 test tubes, beginning with 0 17 ml and then filling up to 0 3 ml by adding antigen solution, by which the amount of antigen would be constant from tube to tube Finally, the haemolytic system was added (0 20 ml per tube), and the results were read after 18 hours at +4° C According to Table 2, 0 15 ml should be used, but to avoid excess of complement 0 13 ml was chosen, corresponding to the complement titre of cardiolipin antigen with the same complement and haemolytic system

TABLE 2

*Determination of Complement Titre Antigen Concentration 0 005 mg per ml C' Dilutions 1 33*

| ml C' 1 1 per tube | 0 1 | 0 15 | 0 12 | 0 11 | 0 10 | 0 08 | 0 07 | 0 06 |
|---|---|---|---|---|---|---|---|---|
| ml antig dil added | 0 13 | 0 15 | 0 18 | 0 19 | 0 20 | 0 22 | 0 23 | 0 24 |
| Eth 50 | 100* | 90 | 70 | 50 | 50 | 30 | 30 | 0 |
| Eth 66 | 100 | 90 | 90 | 70 | 60 | 30 | 20 | 0 |
| Eth 75 | 90 | 90 | 80 | 70 | 60 | 30 | 20 | 0 |

* Per cent haemolysis

After the complement titre had been determined, Palermo 752 was titrated against the 3 antigens in a concentration of 0 005 mg per ml The results are shown in Table 3, and it appears that the greatest reactivity is found in Eth [illegible]

(57 per cent) The [illegible]

in the content of p[illegible]

ments Eth 50 is used therefore as antigen unless otherwise indicated. Using this antigen, the titre for Palermo 752 was very near to 3000.

TABLE 3

*Titration of Serum Palermo 752 against Polysaccharide Antigens*

| Serum dilution | Antigen concentration 0.005 mg per ml | | | | | | | | Serum control |
|---|---|---|---|---|---|---|---|---|---|
| | 1:12 | 1:36 | 1:108 | 1:324 | 1:972 | 1:2916 | 1:8748 | 1:26244 | |
| Eth 50 | 0 | 0* | 0 | 0 | 0 | 60 | 90 | 90 | 100 |
| Eth 66 | 0 | 0 | 0 | 0 | 60 | 90 | 90 | 90 | 100 |
| Eth 75 | 0 | 0 | 0 | 20 | 80 | 90 | 90 | 90 | 100 |

* Per cent haemolysis.

Table 4 shows a preliminary examination of the reactivity of the antigens, the sera being tested in 4 dilutions. The first tube is a control tube containing serum, complement and haemolytic system, but no antigen. The reactivities of the antigens are seen to increase with dilution, and a tendency for the negative serum to react with Eth 50 disappears on dilution.

TABLE 4

*Preliminary Examinations of the Reactivity of the Antigens before the Determination of the Antigen Titre against Immune Serum*

| | Immune serum | | Negative rabbit serum | |
|---|---|---|---|---|
| | Antigen concentration | | Antigen concentration | |
| Antigen | 0.333 mg per ml | 0.005 mg per ml | 0.333 mg per ml | 0.005 mg per ml |
| Eth 50 | 100* 0 0 30 80 | 100 0 0 0 0 | 100 30 50 80 90 | 100 70 80 90 90 |
| *Eth 66* | *100 0 20 90 90* | *100 0 0 0 0* | *100 80 90 90 90* | 100 90 90 90 90 |
| Eth 75 | 100 0 0 30 80 | 100 0 0 0 10 | 100 90 90 90 100 | 100 90 90 90 90 |

* Per cent haemolysis.

The antigen titre was determined by diluting the stock solution, containing 1 mg per ml, with saline and buffered saline (pH 7.38) in the ratio 2 : 1. Immune serum, Palermo 752, was titrated against antigen dilutions in the complement fixation test. The results are shown in Tables 5, 6 and 7.

Thus according to definition the antigen titre is 900 for Eth 50, 600 for Eth 66 and 100 for Eth 75. As expected, the titre is highest for Eth 50 which contains most polysaccharide. Furthermore, zone phenomena are seen with Eth 50 and 66, as there is no change in the reactivity on dilution from 1 : 200 to 1 : 900 and 1 : 600, respectively. The reactivity falls in the range up to 1 : 100 and above 1 : 900 (see also Table 4). In order to avoid working near the limits of the zone of reactivity, a working dilution of 300 *600 has been chosen for Eth 50 and 300 400 for* Eth 66.

TABLE 5

*Cross Titration of Antigen Fth 50 against Serum Palermo 752*

| Antigen dilution | Serum dilution | | | | | | | | Serum control | Antigen control |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 12 | 1 36 | 1 108 | 1 324 | 1 972 | 1 2916 | 1 8748 | 1 26244 | | |
| 1 100 | 0* | 0 | 0 | 0 | 10 | 80 | 90 | 90 | 100 | 90 |
| 1 200 | 0 | 0 | 0 | 0 | × | × | × | × | 100 | × |
| 1 400 | 0 | 0 | 0 | 0 | 10 | 50 | × | × | 100 | × |
| 1 600 | 0 | 0 | 0 | 0 | 10 | 80 | 90 | 90 | 100 | 90 |
| 1 900 | 0 | 0 | 0 | 0 | 20 | 80 | 90 | 100 | 100 | 90 |
| 1 5000 | 80 | 50 | 40 | 50 | 50 | 60 | 80 | 80 | 100 | × |
| 1 10000 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | 90 | × | × |

* Per cent haemolysis × = not done

TABLF 6

*Cross Titration of Antigen Fth 66 against Serum Palermo 752*

| Antigen dilut | Serum dilution | | | | | | | | Serum control | Antigen control |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 12 | 1 36 | 1 108 | 1 324 | 1 972 | 1 2916 | 1 8748 | 1 26244 | | |
| 1 100 | 0* | 0 | 0 | 10 | 60 | 90 | 90 | 90 | 100 | 90 |
| 1 200 | 0 | 0 | 0 | 10 | 50 | 90 | 90 | 90 | × | 90 |
| 1 400 | 0 | 0 | 0 | 10 | 50 | 90 | 90 | 90 | × | 90 |
| 1 600 | 0 | 0 | 0 | 10 | 40 | 90 | 90 | 90 | × | × |
| 1 900 | 10 | 0 | 0 | 10 | 30 | 60 | 90 | 90 | 100 | × |
| 1 1200 | 30 | 10 | 10 | 30 | 90 | 90 | 100 | 100 | × | × |
| 1 2400 | 80 | 40 | 50 | 60 | 90 | 100 | 100 | 100 | × | × |
| 1 4800 | 90 | 90 | 90 | 90 | 100 | 100 | 100 | 100 | × | × |

* Per cent haemolysis × = not done

TABLF 7

*Cross Titration of Antigen Fth 75 against Serum Palermo 752*

| Antigen dilution | Serum dilution | | | | | Serum control |
|---|---|---|---|---|---|---|
| | 1 12 | 1 36 | 1 108 | 1 324 | 1 972 | |
| 1 100 | 0* | 0 | 0 | 20 | 80 | 100 |
| 1 300 | 20 | 10 | 30 | 40 | 60 | × |
| 1 600 | 40 | 40 | 50 | 80 | 90 | × |

* Per cent haemolysis × = not done

Table 8 shows a comparison between the results obtained by two hours' incubation with complement at +4° C plus 30 minutes at 37°C, and those obtained by 18 hours' incubation at +4° C These experiments were made because rabbit sera often were found to be anticomplementary when a prolonged fixation time was employed The reduced haemolysis percentage in the control tube showed that this was a property of the serum In preliminary experiments without serum, it could be demonstrated that complement and haemolytic system were unaffected by the prolonged time of incubation, as the titre of the complement did no change within the period of 18 hours This anticomplemen-

tary effect was not observed, or it was insignificant, using a short fixa tion time, but on the other hand the reactivity was displaced by one dilution step to the left in the series In tube No 1, containing the greatest concentration of serum, a certain inhibition in the complement fixation has been observed with the sera of several rabbits, especially during immunization About the 10th day after the last injection of treponemes, however, 0 per cent haemolysis is found most often in the first tube

TABLE 8

*The Reactivity of Eth 50 after Incubation for 2 hrs at 4° C plus 30 min at 37° C and after 18 hrs at 4° C Performed with 5 Rabbits Immune Sera and 1 Normal Human Serum as Negative Control*

| Serum | | 2 hrs plus 30 min | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Serum dilution | | | | | | | Serum control |
| | | 1 12 | 1 36 | 1 108 | 1 324 | 1 972 | 1 2916 | 1 8748 | |
| Rabbit | 923 | 0 | 0 | 0 | 10 | 60 | 90 | 100 | 100 |
| , | 9593 | 20 | 0 | 0 | 10 | 60 | 90 | 100 | 90 |
| | 9594 | 0 | 0 | 0 | 0 | 10 | 60 | 90 | 100 |
| | 9595 | 20 | 0 | 0 | 0 | 10 | 60 | 90 | 90 |
| , | 9596 | 0 | 0 | 0 | 10 | 60 | 90 | 100 | 100 |
| Normal human | | 100 | | | | | | | 100 |
| | | 18 hrs | | | | | | | |
| Rabbit | 923 | 0 | 0 | 0 | 0 | 10 | 60 | 90 | 80 |
| | 9593 | 0 | 0 | 0 | 0 | 10 | 60 | 90 | 30 |
| | 9594 | 0 | 0 | 0 | 0 | 0 | 10 | 60 | 60 |
| | 9595 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 60 |
| „ | 9596 | 0 | 0 | 0 | 0 | 10 | 40 | 90 | 90 |
| Normal human | | 100 | | | | | | | 100 |

Table 9 shows the results of an examination of the antigenic speci ficity Twenty-four human sera were selected, together with sera from five non-immunized rabbits, and given a qualitative onetube test with antigen Eth 50 On titration human serum 1897 and rabbit serum 573 showed anticomplementary properties Thus the haemolysis value of 20 per cent found for these two sera is not an expression of specific fixation nor does it indicate a presence of non specific properties of the antigen On the contrary, the only reaction found was obtained with immune serum

The specificity of the antigen was further verified by examining a material consisting of 18 cases of primary syphilis, 26 cases of secon dary syphilis, 7 cases of latent syphilis, two cases of lupus erythema-tosus disseminatus, one case of leprosy and finally, serum No 78 from a patient who for several years had shown "biological false positive" STS reactions of unknown cause The material includes both negative and strong positive reactions in STS, and both TPI positive and TPI

negative cases It is observed that the only reaction obtained is that with the specific antiserum As an exception this result is indicated in degrees of strength to permit comparison with STS Also a few syphilitic sera from experimentally infected rabbits were tested but were found to be non reactive

TABLE 9

*Examination of 24 Human Sera and 5 Non Immunized Rabbits*

| Serum | C W R M | KR | MR | Eth 50 |
|---|---|---|---|---|
| Human 1093 | | | | 80 |
| 1248 | 14 | 4 | ± | 80 |
| 1496 | 12 | 9 | ++ | 90 |
| 1539 | 7 | 3 | ± | 90 |
| 1558 | 8 | | × | 60 |
| 1559 | | | | 100 |
| 1560 | | | | 100 |
| 1561 | | | | 100 |
| 1562 | 5 | 2 | — | 100 |
| 1564 | unr § | 1 | × | 100 |
| 1567 | | | | 90 |
| 1590 | 2 | 4 | + | 90 |
| 1594 TPI pos | 13 | 10 | ++ | 90 |
| 1697 | | | | 100 |
| 1759 | | 2 | — | 100 |
| 1760 | | | | 100 |
| 1761 | 4 | | | 100 |
| 1797 | 5 | 1 | | 90 |
| 1804 | 3 | | | 100 |
| 1817 | 1 | 2 | ± | 90 |
| 1827 | 8 | 2 | — | 100 |
| 1897 TPI pos | 20 | 13 | ++ | 20 |
| 2603 | 3 | | | 100 |
| 2[illegible] | 7 | 1 | | 100 |
| Rabbit 96 | | | + | 80 |
| 272 | | | | 40 |
| 295 | | 2 | | 60 |
| 475 | 1 | | | 60 |
| 573 | 1 | | | 20 |
| Antigen control | × | × | × | 90 |
| Immunized rabbit | unr | | | 0 |

Results for Eth 50 in per cent haemolysis
§ unreadable
— not done

Finally the stability of the antigen was tested When Eth 50 and Eth 66 were stored at +4° C both as stock solutions and in dilution with saline no changes were found in the reactivity after two weeks Likewise the reactivity remained unchanged after heating up to 70° C for one hour Stock solutions have been stored at —20° C for up to 4 months without any change in the reactivity The antigen thus possesses the thermostable properties of a polysaccharide

Intravenous injection of antigens Eth 50 and 66 into rabbits in a total amount of 0.8 mg distributed over 6 doses produced demonstrable homologous antibodies in the serum Further it could be shown that

tary effect was not observed, or it was insignificant, using a short fixation time, but on the other hand the *reactivity* was *displaced by one* dilution step to the left in the series. In tube No. 1, containing the greatest concentration of serum, a certain inhibition in the complement fixation has been observed with the sera of several rabbits, especially during immunization. About the 10th day after the last injection of treponemes, however, 0 per cent haemolysis is found most often in the first tube.

TABLE 8

*The Reactivity of Fth 50 after Incubation for 2 hrs. at 4° C plus 30 min. at 37° C and after 18 hrs. at 4° C Performed with 5 Rabbits Immune Sera and 1 Normal Human Serum as Negative Control*

| Serum | | 2 hrs. plus 30 min. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Serum dilution | | | | | | | Serum control |
| | | 1:12 | 1:36 | 1:108 | 1:324 | 1:972 | 1:2916 | 1:8748 | |
| Rabbit | 923 | 0 | 0 | 0 | 10 | 60 | 90 | 100 | 100 |
| „ | *9593* | *20* | *0* | *0* | *10* | *60* | *90* | *100* | *90* |
| „ | 9594 | 0 | 0 | 0 | 0 | 10 | 60 | 90 | 100 |
| „ | 9595 | 20 | 0 | 0 | 0 | 10 | 60 | 90 | 90 |
| „ | 9596 | 0 | 0 | 0 | 10 | 60 | 90 | 100 | 100 |
| Normal human | | 100 | | | | | | | 100 |
| | | 18 hrs. | | | | | | | |
| Rabbit | 923 | 0 | 0 | 0 | 0 | 10 | 60 | 90 | 80 |
| „ | 9593 | 0 | 0 | 0 | 0 | 10 | 60 | 90 | 30 |
| „ | 9594 | 0 | 0 | 0 | 0 | 0 | 10 | 60 | 60 |
| „ | 9595 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 60 |
| „ | 9596 | 0 | 0 | 0 | 0 | 10 | 40 | 90 | 90 |
| *Normal human* | | *100* | | | | | | | *100* |

Table 9 shows the results of an examination of the antigenic specificity. Twenty-four human sera were selected together with sera from *five non-immunized rabbits, and given a qualitative onetube test with* antigen Fth. 50. On titration, human serum 1897 and rabbit serum 573 showed anticomplementary properties. Thus the haemolysis value of 20 per cent found for these two sera is not an expression of specific fixation, nor does it indicate a presence of non-specific properties of the antigen. *On the contrary, the only reaction found was obtained with* immune serum.

The specificity of the antigen was further verified by examining a material consisting of 18 cases of primary syphilis, 26 cases of secondary syphilis, 7 cases of latent syphilis, two cases of lupus erythematosus disseminatus, one case *of leprosy and finally, serum No. 78 from* a patient who for several years had shown "biological false positive" STS reactions of unknown cause. The material includes both negative and strong positive reactions in STS and both TPI positive and TPI

Eth 50, in addition to reacting with its homologous serum, also reacted with serum from the rabbit immunized with Eth 66, and vice versa Eth 7a reacted with both these sera, but more weakly, as expected This indicates that the 3 substances contain at least one common prosthetic group with respect to the antigenic properties The highest antibody content was found in serum from a rabbit immunized with Eth 50 in agreement with the fact that this substance has the highest content of polysaccharide

## DISCUSSION

Polysaccharide has been isolated from Reiter treponemes by means of the phenol water method (2, 16), and it has been shown that this polysaccharide reacts with homologous immune seera in complement fixation tests The polysaccharide is active in solutions containing at least 1 gamma per ml, and the reactivity does not decline on standing at +4° C or heating to 70 C for one hour

The polysaccharide isolated by Professor *D Alessandro et al* (3) in 1949 is reported as being reactive with immune serum in an antigen dilution of 1 19200 of a stock solution containing 3 mg of polysaccharide per ml, i e 0 15 gamma per ml antigen dilution This polysaccharide was isolated by treatment of Reiter treponemes with N NaOH, followed by neutralization with trichloracetic acid and precipitation with ethanol The polysaccharide isolated by the phenol-water method has to be present in an about 10 times higher concentration in order to react with immune sera The reason for this difference in reactivity may be that the D Alessandro method causes a partial hydrolysis of the polysaccharide but with the prosthetic groups a retained, so that the antigen has the characteristics of a haptene, but cannot function as a complete antigen The phenol water method avoids strong acids and bases In this way a polysaccharide complex is obtained about one half of which consists of polysaccharide In addition, there is a small amount of protein and some still unanalyzed material which may be a lipid The polysaccharide complex is a complete antigen which when injected into rabbit causes production of homologous antibodies in analogy with [illegible] 16) Later, *D Alessan*[illegible] from Reiter trepone[illegible] [illegible]s This antigen, however also reacted with syphilis sera (76 out of 85 were positive), in contrast with the polysaccharide complex isolated by the phenol water method This polysaccharide has not reacted with a single serum from the 51 patients with various stages of syphilis The antigen isolated by *D Alessandro & DelCarpio*, therefore, must posses other constituents besides polysaccharide, possibly protein

*Pillot et al* (12) prepared a polysaccharide from [illegible]

TABLE 10

*Examination of Sera from 51 Patients with Lues in Various Stages and 5 Patients with Biological False Positive Reactions*

| Serum no | Diagnosis | STS* | TPI | Tith 50 in C fix | Serum no | Diagnosis | STS | TPI | Tith 50 in C fix | Serum no | Diagnosis | STS | TPI | Tith 50 in C fix |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 18 | Lues I | 8 5 + | | neg | 20 | Lues II | 19/12 ++ | + | neg | 72 | Lues II | 5/3 — | + | neg |
| 25 | I | 12 6 ++ | + | | 24 | II | 11/ 8 + | + | | 74 | II | 13/9 ++ | + | |
| 31 | I | 13 9 ++ | ± | | 26 | II | 15/ 9 ++ | + | | 82 | II | 11/7 ++ | × | |
| 33 | I | | | | 30 | II | / — | + | | 83 | II | 10/6 ++ | + | |
| 36 | I | 2/2 + | | | 32 | II | 14/ 9 ++ | + | | 84 | II | 11/8 ++ | × | |
| 37 | I | 8 3 + | + | | 44 | II | 12/ 6 ++ | × | | 86 | II | 10/6 + | + | |
| 38 | I | 5 3 + | ± | | 45 | II | 11/ 9 ++ | + | | 8 | lat | 4/2 ± | + | |
| 40 | I | 4/2 + | × | | 46 | II | 11/ 7 + | × | | 11 | lat | / — | × | |
| 41 | I | 8 6 ++ | | | 47 | II | 11/10 ++ | × | | 34 | lat | ?/ ± | + | |
| 42 | I | 8/5 + | | | 48 | II | 11/ 9 ++ | ± | | 35 | lat | 7/6 ++ | + | |
| 52 | I | 13 9 ++ | | | 49 | II | 11/ 6 ++ | + | | 67 | lat | 8/7 + | + | |
| 53 | I | 7/6 ++ | | | 50 | II | 11/ 9 + | + | | 69 | lat | 8/6 ± | + | |
| 54 | I | 8/6 + | + | | 51 | II | 12/ 7 + | + | | 73 | lat | 11/9 ++ | + | |
| 58 | I | 7 3 + | + | | 55 | II | 8/ 6 ++ | ± | | 27 | LFD* | 1/1 + | — | |
| 59 | I | 11/9 ++ | | | 57 | II | 4/ 2 | — | | 29 | LFD obs | / + | — | |
| 68 | I | | | | 62 | II | 6/ 1 — | — | | 75 | Lepra | 12/unr — | — | |
| 76 | I | 6/3 + | × | | 63 | II | 1/ 1 | — | | 78 | No lues | 8/ + | — | |
| 77 | I | 4 | | | 65 | II | 5/ 3 ± | — | | 923 | Imm serum | unr /- — | × | 16† |
| 16 | II | 1 /5 + | + | | 66 | II | 12/ 9 ++ | + | | | | | | |

* STS = CWR MKR MR
* Lupus erythematosus disseminatus
† Degrees of strength
neg = negative  × = not done  unr = unreadable

demonstrated between the polysaccharide fractions Eth 50, 66 and 75 Only a quantitative difference between these fractions could be demonstrated, corresponding to the polysaccharide content

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saccharide per ml, and in dilutions of 1 : 320 it reacted with immune serum diluted 1 : 25 The polysaccharide complex isolated by the phenol-water method contains 3 times less material in the same dilution, but nevertheless it reacts with immune serum diluted 3000 times, and the reactivity is practically unchanged in an antigen dilution of 1 : 900

*De Bruijn* (6) prepared polysaccharide from Reiter protein antigen by trypsin treatment This polysaccharide reacted with 12 out of 205 syphilitic sera examined, and reacted with serum from rabbits immunized with Reiter protein antigen This antigen was shown to have a *termolabile component in low dilutions, considered to be of a lipid nature* Traces of protein, however, could also be detected, but this cannot explain the reactivity with the 12 syphilitic sera, as the antigen used had been heated for one hour at 100° C Comparing Tables 1 and 3, it is seen that serum Palermo 752 had a titre between 320 and 640 when tested with Reiter protein antigen, but that the serum titre was 2816 when tested with Eth 50 as antigen The polysaccharide thus has a considerably greater reactivity The high reactivity of the polysaccharideantigen makes a long fixation time unnecessary, so that the original 18 hours' fixation could be reduced to 2½ hours The advantage of the reduction in fixation time is that such curtailed fixation time reduces chances of a development of the haemolysis-inhibiting property of immune serum from rabbits, a phenomenon not infrequently observed using the prolonged fixation time, (Table 8)

The present studies confirmed the theory of the high specificity of the polysaccharide, as a reaction was obtained only with serum from rabbits immunized with Reiter treponemes or polysaccharide isolated from these A reaction with syphilitic sera from rabbit or humans has not been observed

Cross reactions suggested that Eth 50, 66 and 75 reacted with the same antibody Furthermore, a quantitative difference could be demonstrated between these fractions, corresponding to the polysaccharide content Haemolysis-inhibiting properties of the antigen were not observed with the usual antigen concentrations of about 3 gamma per ml, but on the other hand they were seen with concentrations of about 300 gamma per ml (Table 4)

## SUMMARY

The polysaccharide complex isolated from Reiter treponemes by the phenol water method, has antigenic properties, as it reacts with homologous immune sera and induces the production of antibodies on injection into rabbits No reactions have been seen with syphilitic sera from humans or rabbits

The results obtained by other authors are discussed in relation to the present results

Immunological differences of a fundamental nature could not be

demonstrated between the polysaccharide fractions Fth 50, 66 and 75 Only a quantitative difference between these fractions could be demonstrated corresponding to the polysaccharide content

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# ACID MUCOPOLYSACCHARIDES IN MICROCOCCUS (STAPHYLOCOCCUS AUREUS)

*By*

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Received 13 iv 62

Immunological, reactive polysaccharides have been described in sero logical active staphylococci Thus *Julianelle & Wieghard* (1935 a & b) and *Wieghard & Julianelle* (1935) described two different polysaccharides (A and B) occurring in pathogenic and non-pathogenic strains of staphylococci This caused *Cowan* (1938) to divide the pathogenic staphylococci into three groups Furthermore *Hoffstadt & Clark* (1935) described two different polysaccharides and *Jensen* (1959) demonstrated that polysaccharide A occurred in Cowan group I Apart from the identification of N-acetylaminohexose in H lysodeikticus (*Epstein & Chain* 1940) and glucose, mannose, glucosamine and glucuronic acid in the same bacteria (*Hawthorne* 1950) no trial has been performed to characterize the polysaccharides in different groups of staphylococci by chemical and biochemical methods, especially to differentiate between acid mucopolysaccharides and other polysaccharides In this paper, data are given for the occurrence of acid mucopolysaccharides in the three groups of Cowan

## MATERIAL AND METHODS

The three groups Cowan I II and III were objects of the investigations (fag group I II and III) The bacterias were cultivated on agar bouillon for 24 hours The culture was scraped off and washed three times with saline and each time the bacterias were again isolated by centrifugation (10 000 rpm) The culture from one plate was resuspended in 10 ml of buffer (9 ml $^1/_{15}$ M $KH_2PO_4$ + 1 ml $^1/_{15}$ M $Na_2HPO_4$) and by standing for 14 days at room temperature most of the soluble material was now in the supernatant (*Jensen* 1959) The remnants of the cell bodies were taken away by centrifugation The supernatant was concentrated 10 times in an apparatus for vacuum dialysis (Clausen 1962)

The supernatant was controlled for its content of acid mucopolysaccharides by electrophoresis on cellulose acetate paper in a modified LKB apparatus followed by specific staining for acid mucopolysaccharides with mucicarmine The LKB ap[illegible] replacing the fakir [illegible]lter paper connec[illegible] foam rubber made it possible to use cellulose acetate paper [illegible] with excellent results The electrophoresis was performed in veronal buffer (pH = 8 6 ionic strength = 0 25) for 1 ½ hour of 10 μl supernatant (200 v) and after drying on glass plates at

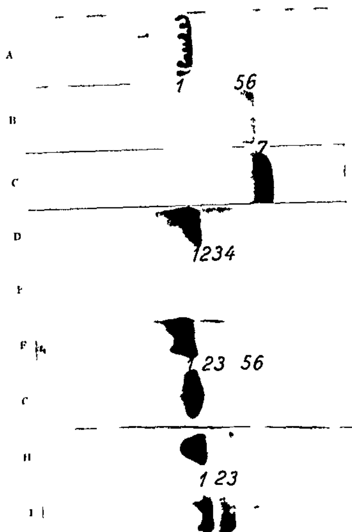



*Fig 1*

Acid mucopolysaccharides pattern on cellulose acetate paper of extractable components from Staphylococci (Cowan group I, II and III) correlated to standard preparations of hyaluronic acid, chondroitinsulphate and heparine

A Hyaluronic acid (containing impurities of chondroitinsulphate)
B Chondroitin sulphate A + B
C Heparine
D Pooled fractions from Cowan group I, II and III
E Cowan group I
F Pooled fractions from Cowan group I, II and III
G Cowan group II
H Pooled fractions from Cowan group I, II and III
I Cowan group III

110° C the cellulose acetate paper was stained for 10 minutes with mucicarmine (1 g per cent W/V in 50 per cent ethanol) The background was decolorized with 10 per cent acetic acid Finally the paper was dried under pressure between two parts of filter paper The electrophoretic patterns of the acid mucopolysaccharides were correlated to the patterns of standard solutions of heparine (NOVO), chondroitinsulphate (Sigma) and hyaluronic acid (The Serum Institute Copenhagen)

## RESULTS

Figure 1 illustrates the results In the pooled mixture of the extracts from the three groups, 6 acid mucopolysaccharide fractions can be visualized (fraction 1–6) The three most anodic ones have mobilities as chondroitinsulphate, but no one had the same mobility as heparine On the other hand the most cathodic fraction had the mobility corresponding to hyaluronic acid In the intermediate area two more fractions can be visualized, but none of these correspond in mobility to any of the standard fractions

By comparison of the extract of the single groups no one of these possessed all of the fractions seen in the pooled preparation Thus the Cowan group I does not have the slowest moving fraction, with a mobility like that of hyaluronic acid (fraction I) Furthermore, the two most anodic fractions are also lacking in this group (fraction 5 and 6 with mobility as chondrosulphate A and B) These two fractions of acid mucopolysaccharides with the highest mobility are present only in Cowan group II This group contains obviously all of the acid mucopolysaccharide fractions with the exception of one of the intermediate fractions (fraction 4), which is present in Cowan group I Cowan group III contains the fractions 1–3, but seems to lack the fractions 4–6, probably the group of chondroitinsulphates

## DISCUSSION

Among the acid mucopolysaccharides most investigations concerning bacteria have been centered around the presence of hyaluronic acid Thus early investigations seem to indicate that hyaluronic acid is present in streptococci, but this compound does not possess antigenic properties (*Quinn & Singh* 1957 *Seifter Baeder, Beckfield* 1954) Isotope studies furthermore, have revealed in streptococci the pathway for synthesis of hyaluronic acid (cit *H Gibian* 1959) Finally the content of hyaluronic acid in bacteria seem probably to depend on the content of hyaluronidase because penicillin seems to increase the amount of hyaluronic acid (cit *H Gibian* 1959) Thus the existence of hyaluronic acid in bacterias seems to be a well established fact, especially in streptococci The present investigations confirm that hyaluronic acid also exist in some groups of staphylococci, but that hyaluronic acid is lacking in at least one of the groups (Cowan I), which is probably characteristic of this group

Likewise the existence of a group of acid mucopolysaccharides with a mobility like that of the group of chondroitinsulphates seems to be variable from group to group of staphylococci Thus Cowan I and III are lacking in this group, but chondroitinsulphate is present in Cowan II As is the case of hyaluronic acid antigen properties are claimed to be absent also in the group of chondroitinsulphates where as they can react with proteins in $\beta$-haemolytic streptococci forming an auto-antigen (*Glynn & Holborow* 1952), but this has not been accepted by later investigations (*Glynn & Johnson* 1956) Thus it remains still to be demonstrated which of the immunological properties can be attributed to acid mucopolysaccharides, especially because *Julianelle & Wieghard* (1935) could use the polysaccharides in staphylococci by a precipitation reaction for a division of the staphylococci into different groups But it is possible that the polysaccharides type A and B do not represent *acid* mucopolysaccharides On the other hand it has to be mentioned that some of the acid mucopolysaccharides, by us demonstrated to be common in the three Cowan groups (*e g* fraction 2) could represent, perhaps partially, the polysaccharide A which by *Jensen* (1959) was demonstrated in 75 per cent of a series of groups, and, furthermore, investigations by one of us (*Rosenkast* 1962) seem to indicate even a higher incidence of polysaccharide A than the one shown by *Jensen*

## SUMMARY

By means of a new method, based upon an electrophoretic separation on cellulose acetate paper in a modified LKB apparatus, it was possible by a specific staining procedure with mucicarmine to demonstrate six acid mucopolysaccharide fractions with different mobility in Staph aur Cowan's groups I, II and III The fractions differed from one group to the other, thus demonstrating the occurrence of acid mucopolysaccharides to be characteristic of different groups of staphylococci

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FROM THE TUBERCULOSIS AND STATISTICAL DEPARTMENTS
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# ACQUIRED RESISTANCE TO TUBERCULOSIS AND TUBERCULIN SENSITIVITY IN GUINEA PIGS VACCINATED WITH A SMALL OR A LARGE DOSE OF BCG VACCINE

By

ANDR JESPERSEN, M WEIS BENTZON and
MOGENS MAGNUSSON

Received 2 III 62

In a previous report (*Jespersen* 1956) the tuberculin sensitivity and acquired resistance to tuberculosis evoked by subcutaneous or intraperitoneal vaccination of guinea pigs with varying doses ($10^{-6}$–$10^{-1}$ mg) of BCG vaccine were studied The vaccination period was six to seven weeks It was found that $10^{-6}$ mg BCG vaccine had scarcely any immunizing effect A dose of 10 [illegible] mg vaccine induced tuberculin sensitivity in approximately 50 per cent of the animals and the survival time was also prolonged 10 [illegible] mg vaccine immunized practically all of the animals Though the survival time was prolonged when increasing doses of vaccine were used, there was no pronounced difference in the degree of acquired resistance between the $10^{-1}$ mg group and the tuberculin positive animals in the 10 [illegible] mg group In a later study (*Jespersen et al* 1962) the tuberculin sensitivity and immunity seemed to increase in guinea pigs vaccinated subcutaneously with 10 [illegible] mg BCG up to six to eight weeks after vaccination, and in this dose range both seemed to be influenced even by fairly small variations in the vaccine dose

The aim of the present study was to see whether the acquired resistance and tuberculin sensitivity induced by vaccination with a large ($10^{-1}$ mg) and a small (10 [illegible] mg) dose of BCG vaccine were different for longer vaccination periods The vaccination period varied from 1½ months to 9 months, and both subcutaneous and intracutaneous methods of vaccination were used

Furthermore, it was desired to investigate whether the duration of the tuberculin sensitivity and acquired resistance in BCG vaccinated guinea pigs [illegible]
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# ACQUIRED RESISTANCE TO TUBERCULOSIS AND TUBERCULIN SENSITIVITY IN GUINEA PIGS VACCINATED WITH A SMALL OR A LARGE DOSE OF BCG VACCINE

By

ANDR. JESPERSEN, M. WEIS BENTZON and MOGENS MAGNUSSON

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The aim of the present study was to see whether the acquired resistance and tuberculin sensitivity induced by vaccination with a large ($10^{-1}$ mg) and a small ($10^{-5}$ mg) dose of BCG vaccine were different for longer vaccination periods. The vaccination period varied from 1½ months to 9 months, and both subcutaneous and intracutaneous methods of vaccination were used.

Furthermore, it was desired to investigate whether the duration of the tuberculin sensitivity and acquired resistance in BCG vaccinated guinea pigs is dependent on the persistence of viable BCG bacteria in the host. Efforts were thus made to see whether BCG

## EXPERIMENTAL

Groups of guinea pigs were vaccinated at different times (see Table 1) with doses of $10^{-5}$ mg and $10^{-1}$ mg BCG. The vaccinations were timed so that the period from vaccination to challenge (performed on the same day for all the animals) was 1½, 3, 6 or 9 months. Seven days before challenge all of the vaccinated animals and three non-vaccinated control groups were given intradermal tuberculin tests with 5 *tuberculin units (TU)* and four days before challenge with 250 TU. All of the animals were challenged with a small dose of virulent bovine tubercle bacilli given intravenously, except for two (or four) from each of the vaccinated groups which were killed and examined for the presence of BCG bacteria. The animals were observed until they died of the tuberculous infection. The survival time in days after *day of challenge was used as indication of the acquired resistance.*

## MATERIALS AND METHODS

*Animals.* The animals used were albino female guinea pigs bred at the farm attached to Statens Seruminstitut. At the commencement of the study, *i.e.* before vaccination, all of the animals weighed 250 to 300 gr. They were fed on hay, corn mixture, and sugar beet or kohlrabi. Two guinea pigs were kept in each cage.

At the time of challenge the vaccinated guinea pigs varied in age from 3 to 11 months. Three non-vaccinated control groups of animals aged 3, 7 and 11 months were therefore included in the study. These animals were all males, since it was not possible at that time to procure an adequate number of female albino guinea pigs. In addition, a non-vaccinated control group of female guinea pigs was included. However, this latter group was not tuberculin tested before challenge as were the other animals.

None of the guinea pigs showed any reaction to a pre-vaccination tuberculin test with 250 TU.

*Vaccination with BCG vaccine.* Freshly prepared liquid BCG vaccine from the BCG Department, Statens Seruminstitut, containing 0.75 mg of BCG (semidried weight) per ml, was diluted to a concentration of $10^{-1}$ mg or $10^{-5}$ mg per 0.2 ml.[1] The diluent was diluted Sauton medium (one part Sauton medium and three parts distilled water). Each animal was vaccinated subcutaneously in the right inguinal region with 0.2 ml vaccine, or intracutaneously with 0.1 ml per site on two sites. These two injections were made on a line down the middle of the abdomen, the one near the lower part of the thorax and the other on the lower part of the abdomen. The vaccines were protected against light during preparation and use, and vaccination was carried out by artificial light.

Colony counts of the vaccines were made after inoculation on Lowenstein-Jensen medium, 0.1 ml on each of ten tubes, with samples of the freshly prepared vaccine ("before vaccination") and vaccine exposed to the same conditions as the vaccine used ("after vaccination"). For two of the vaccines the colony counts were slightly smaller after vaccination than before (see Table 1).

On the basis of the colony counts (Table 1), the vaccination doses were approximately 120 viable units ($10^{-5}$ mg) or 1 200 000 viable units ($10^{-1}$ mg), except for animals vaccinated 1½ months before challenge where the doses were lower (approximately 60 and 600 000 viable units respectively).

*Tuberculin testing.* Seven days before challenge the animals were given two intradermal tuberculin injections of 0.1 ml, one on each side of the upper part of the back, using Purified Tuberculin (PPD) Batch RT 22, Statens Seruminstitut. The dose was 5 TU (0.067 μg) per 0.1 ml and the diluent phosphate buffered saline with 0.01 per cent chinosol.

The reactions were read after 24 hours by two independent readers. The animals were taken cage-wise in random order, the readers having no knowledge of the groups to which these belonged. The mean value of two diameters of erythema at right angles to each other was recorded. The results given are the averages for the two readers, using the mean of the two reactions on each animal.

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[1] The authors are grateful to Miss *K. Bunch Christensen, BCG Department, Statens Seruminstitut*, for preparing and supplying the vaccine.

TABLE 1

*Guinea Pig Vaccination Schedule*

| Vaccination period | Vaccination date | No. of animals vaccinated § | | | | BCG vaccine | | | Vaccination dose † (viable units) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Subcutaneous vaccination | | Intracutaneous vaccination | | SSI lot no. | Colony counts (viable units) per 0.75 mg | | 10[illegible] mg | 10[illegible] mg |
| | | 10[illegible] mg | 10[illegible] mg | 10[illegible] mg | 10[illegible] mg | | Before vacc. | After vacc. | | |
| 1½ months | 21 1 1957 | 24 | 24 | 24 | 24 | 1242 | $0.5 \times 10^7$ | $0.5 \times 10^7$ | 60 | 600 000 |
| 3 | 18 12 1956 | 24 | 24 | 24 | 24 | 1236 | $0.8 \times 10^7$ | $0.9 \times 10^7$ | 110 | 1 100 000 |
| 6 | 18 9 1956 | 27 | 27 | 27 | 27 | 1223 | $1.1 \times 10^7$ | $0.9 \times 10^7$ | 130 | 1 300 000 |
| 9 | 20 6 1956 | 30 | 30 | | | 1210 | $1.0 \times 10^7$ | $0.6 \times 10^7$ | 110 | 1 100 000 |

§ Larger groups were vaccinated for vaccination periods 6 and 9 months to compensate for an expected spontaneous mortality of animals in the vaccination period.
† Estimated from means of colony counts before and after vaccination.

## EXPERIMENTAL

Groups of guinea pigs were vaccinated at different times (see Table 1) with doses of $10^{-5}$ mg and $10^{-1}$ mg BCG. The vaccinations were timed so that the period from vaccination to challenge (performed on the same day for all the animals) was 1½, 3, 6 or 9 months. Seven days before challenge all of the vaccinated animals and three non-vaccinated control groups were given intradermal tuberculin tests with 5 *tuberculin units (TU)* and four days before challenge with 250 TU. All of the animals were challenged with a small dose of virulent bovine tubercle bacilli given intravenously, except for two (or four) from each of the vaccinated groups which were killed and examined for the presence of BCG bacteria. The animals were observed until they died of the tuberculous infection. The survival time in days after day of challenge was used as indication of the acquired resistance.

## MATERIALS AND METHODS

*Animals.* The animals used were albino female guinea pigs bred at the farm attached to Statens Seruminstitut. At the commencement of the study, i.e. before vaccination, all of the animals weighed 250 to 300 gr. They were fed on hay, corn mixture and sugar beet or kohlrabi. Two guinea pigs were kept in each cage.

At the time of challenge the vaccinated guinea pigs varied in age from 3 to 11 months. Three non-vaccinated control groups of animals aged 3, 7 and 11 months were therefore included in the study. These animals were all males, since it was not possible at that time to procure an adequate number of female albino guinea pigs. In addition, a non-vaccinated control group of female guinea pigs was included. However, this latter group was not tuberculin tested before challenge as were the other animals.

None of the guinea pigs showed any reaction to a pre-vaccination tuberculin test with 250 TU.

*Vaccination with BCG vaccine.* Freshly prepared liquid BCG vaccine from the BCG Department, Statens Seruminstitut, containing 0.75 mg of BCG (semidried weight) per ml, was diluted to a concentration of $10^{-1}$ mg or $10^{-5}$ mg per 0.2 ml.[1] The diluent was diluted Sauton medium (one part Sauton medium and three parts distilled water). Each animal was vaccinated subcutaneously in the right inguinal region with 0.2 ml vaccine or intracutaneously with 0.1 ml per site on two sites. These two injections were made on a line down the middle of the abdomen, the one near the lower part of the thorax and the other on the lower part of the abdomen. The vaccines were protected against light during preparation and use, and vaccination was carried out by artificial light.

Colony counts of the vaccines were made after inoculation on Lowenstein-Jensen medium, 0.1 ml on each of ten tubes, with samples of the freshly prepared vaccine ("before vaccination") and vaccine exposed to the same conditions as the vaccine used ("after vaccination"). For two of the vaccines the colony counts were slightly smaller after vaccination than before (see Table 1).

On the basis of the colony counts (Table 1) the vaccination doses were approximately 120 viable units ($10^{-5}$ mg) or 1 200 000 viable units ($10^{-1}$ mg), except for animals vaccinated 1½ months before challenge where the doses were lower (approximately 60 and 600 000 viable units respectively).

*Tuberculin testing.* Seven days before challenge the animals were given two intradermal tuberculin injections of 0.1 ml, one on each side of the upper part of the back, using Purified Tuberculin (PPD) Batch RT 22, Statens Seruminstitut. The dose was 5 TU (0.067 µg) per 0.1 ml and the diluent phosphate buffered saline with 0.01 per cent chinosol.

The reactions were read after 24 hours by two independent readers. The animals were taken cage-wise in random order, the readers having no knowledge of the groups to which these belonged. The mean value of two diameters of erythema at right angles to each other was recorded. The results given are the averages for the two readers, using the mean of the two reactions on each animal.

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[1] The authors are grateful to Miss *K. Bunch Christensen, BCG Department, Statens Seruminstitut,* for preparing and supplying the vaccine.

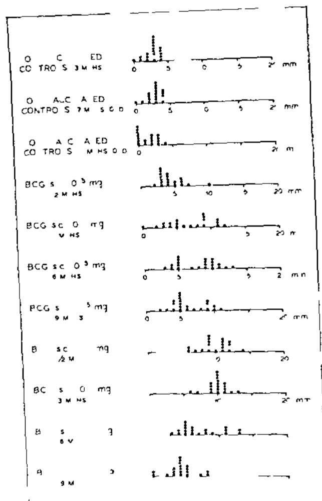


*Fig. 1*

Distribution of tuberculin reactions (erythema in mm) to 5 TU of non vaccinated guinea pigs and guinea pigs vaccinated subcutaneously with 10 mg or $10^{-1}$ mg BCG vaccine at specified periods before testing

*Intracutaneous* vaccination with *$10^{-5}$ mg of BCG* caused the tuberculin sensitivity to develop more quickly and the reactions were larger (Fig. 2) as compared to subcutaneous vaccination with the same dose. The mean size of reactions reached its maximum after three months (11.3 mm) (Table 2). At that time all of the animals in the group had larger tuberculin reactions than the control animals. After six months the sensitivity had waned and one or two guinea pigs had completely lost their sensitivity to 5 TU. The decrease in the tuberculin sensitivity between three and six months was not due to differences in vaccine dosage (see Table 1).

Four days before challenge the animals were tested in the same way with a larger dose—250 TU (3 3 μg)—*of RT 22 per 0 1 ml* The two injections with this dose were given on each side of the *lower part of the back*

*Isolation of BCG bacteria from vaccinated animals* At the time of challenge two *or four guinea pigs* were selected at random from each of the vaccinated groups They were anaesthetized with ether, and the inguinal *lymph node in the right groin, the* corresponding lumbar lymph node, half the spleen, and a piece of liver about 1 cm³ in size, were removed by sterile section The individual organs were *placed in a* sterile porcelain mortar and crushed by vigorous grinding for five minutes while about 1 ml of diluted Sauton medium was added The organ suspensions were inoculated on to five Lowenstein Jensen tubes without prior treatment with sodium hydroxide The tubes were placed in incubator at 37° and the results of culture read six weeks later

*Tissue specimens were taken at the autopsy from the spleen, liver and lungs for* histological examination The organs were fixed in formalin, sectioned and stained with haematoxylin-eosin and also by the Ziehl-Neelsen method for examination for tubercle bacilli

*Challenge* The animals were challenged with a suspension of virulent bovine tubercle bacilli strain E 6884 B A suspension containing 5 × 10⁻⁶ mg of bacilli (semi-dried weight) per ml was prepared in diluted Sauton medium using an 11 day-old Besredka culture Viable counts on Lowenstein-Jensen medium showed that the suspension contained approximately 225 bacterial units per ml

Each animal was injected intravenously into an ear vein with 0 2 ml of bacterial suspension *i e* 40 50 viable bacterial units The animals were taken cage wise in *random order and the challenge process took eight hours to perform* The bacterial suspension was protected against daylight during preparation and use Bacterial counts on the suspension before and after use showed that a slight fall in the number of viable units in the challenge dose had occurred during use the decrease being from approximately 45 viable units at the beginning to 25 to 30 viable units at the end of the process However, graphical evaluation showed that there was no relationship between the order in which challenge was carried out and the length of the survival time

The animals were allowed to die spontaneously The extent of the tuberculous infection on post mortem examination was classified as Tub 0 to Tub V as described previously (*Jespersen* 1954) The survival time from day of challenge for *the animals with moderate or severe generalized tuberculosis* (Tub IV or Tub V) was used as indication of the acquired resistance

## RESULTS

### *Tuberculin sensitivity*

*Reactions to 5 TU* The reaction to 5 TU was less than 5 mm in all of the *non-vaccinated* animals (Fig 1) The mean reactions for the 3 to 7 month-old animals were 2 4 mm and for the 11 month-old 1 3 mm (Table 2)

The majority of the guinea pigs vaccinated *subcutaneously* with 10⁻¹ *mg BCG vaccine* gave larger reactions (Fig 1) than the non-vaccinated On the other hand, in each of the four groups vaccinated with this dose were a few animals without any tuberculin sensitivity The mean reaction increased from 4 1 mm at 1½ months to 7 9 mm at six months (Table 2) At nine months the mean reaction had decreased to 6 3 mm

Subcutaneous vaccination with *10⁻¹ mg of BCG vaccine* caused the tuberculin sensitivity to develop more quickly, but it also waned more quickly (Fig 1) The mean reaction (Table 2) reached its maximum after three months (9 6 mm) and had decreased considerably after nine months (4 6 mm) By that time about half of the animals had lost their tuberculin sensitivity when tested with 5 TU

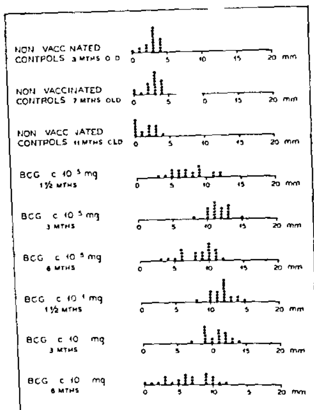



*Fig 2*

Distribution of tuberculin reactions (erythema in mm) to 5 TU of non vaccinated guinea pigs and guinea pigs vaccinated intracutaneously with 10 5 mg or 10 1 mg BCG vaccine at specified periods before testing

*Reactions to 250 TU* The distribution of the reactions to 250 TU is shown in Figs 3 and 4 (see also Appendix Tables 1, 2, and 3) As expected the reactions to 250 TU are larger than to 5 TU in all of the animals However, the difference in reactions to the two doses is smaller in the controls than in the vaccinated animals In particular, some of the vaccinated animals which had lost their sensitivity to 5 TU show fairly strong reactions to 250 TU Thus a better separation between vaccinated and non-vaccinated guinea pigs is obtained by the 250 TU test

By and large, the mean reactions to 250 TU (Table 3) vary in the same way as the reactions to 5 TU as far as the influence of the vaccination period, the vaccination dose and the vaccination route, are concerned

Thus the present study has shown that the tuberculin sensitivity develops more quickly, reaches a higher maximum level, and begins to wane more quickly in guinea pigs vaccinated *intracutaneously* with 10 5

TABLE 2

*Mean Size of Tuberculin Reactions (in mm) to Intradermal Tests with 5 TU of Non-Vaccinated Guinea Pigs and Guinea Pigs Vaccinated with BCG Vaccine at Specified Periods before Testing (Each Animal Given two Tests)*

| Group | | Subcutaneous vaccination | | | Intracutaneous vaccination | | |
|---|---|---|---|---|---|---|---|
| Vaccine dose | Vaccination period months | No of animals | Mean size of tuberculin reaction | Standard deviation | No of animals | Mean size of tuberculin reaction | Standard deviation |
| $10^{5}$ mg BCG | 1½ | 19 | 4 1 | 1 9 | 21 | 7 3 | 2 6 |
| | 3 | 21 | 6 5 | 3 2 | 21 | 11 3 | 1 4 |
| | 6 | 23 | 7 9 | 3 1 | 24 | 8 3 | 2 4 |
| | 9 | 25 | 6 3 | 2 5 | – | – | – |
| $10^{1}$ mg BCG | 1½ | 22 | 9 5 | 2 1 | 22 | 11 4 | 1 6 |
| | 3 | 21 | 9 6 | 1 8 | 21 | 10 3 | 1 6 |
| | 6 | 23 | 7 1 | 2 8 | 25 | 6 3 | 3 3 |
| | 9 | 26 | 4 6 | 2 6 | – | – | – |
| Non vaccinated controls | | | | | | | |
| 3 months old | | 20 | 2 4 | 1 1 | | | |
| 7 „ „ | | 20 | 2 3 | 1 1 | | | |
| 11 „ | | 18 | 1 3 | 1 3 | | | |

Intracutaneous vaccination with $10^{1}$ *mg of BCG vaccine* caused the tuberculin sensitivity to reach its maximum already after 1½ months when the mean size of reactions was 11 4 mm (Table 2) After six months the mean reaction had decreased to 6 3 mm (Table 2), and approximately one third of the animals had lost their sensitivity to 5 TU (Fig 2)

TABLE 3

*Mean Size of Tuberculin Reactions (in mm) to Intradermal Tests with 250 TU of Non Vaccinated Guinea Pigs and Guinea Pigs Vaccinated with BCG Vacine at Specified Periods before Testing (Each Animal Given two Tests)*

| Group | | Subcutaneous vaccination | | | Intracutaneous vaccination | | |
|---|---|---|---|---|---|---|---|
| Vaccine dose | Vaccination period months | No of animals | Mean size of tuberculin reaction | Standard deviation | No of animals | Mean size of tuberculin reaction | Standard deviation |
| $10^{5}$ mg BCG | 1½ | 19 | 6 6 | 3 6 | 21 | 14 0 | 3 1 |
| | 3 | 21 | 11 9 | 3 6 | 21 | 16 0 | 1 1 |
| | 6 | 23 | 13 1 | 3 3 | 24 | 14 5 | 1 8 |
| | 9 | 25 | 11 1 | 5 4 | | | |
| $10^{1}$ mg BCG | 1½ | 22 | 16 8 | 1 7 | 22 | 17 0 | 1 4 |
| | 3 | 21 | 15 8 | 1 4 | 21 | 15 8 | 1 5 |
| | 6 | 23 | 13 4 | 2 3 | 25 | 14 0 | 2 2 |
| | 9 | 26 | 11 0 | 3 1 | | | |
| Non vaccinated controls | | | | | | | |
| 3 months old | | 20 | 3 5 | 0 8 | | | |
| 7 „ | | 20 | 3 0 | 1 3 | | | |
| 11 „ | | 19 | 3 1 | 2 0 | | | |

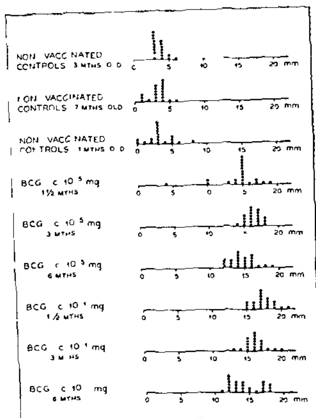



*Fig 4*

Distribution of tuberculin reactions (erythema in mm) to 250 TU of non vaccinated guinea pigs and guinea pigs vaccinated intracutaneously with $10^{-5}$ mg or $10^{-1}$ mg BCG vaccine at specified periods before testing

*Survival times*

During the vaccination period 8 out of the 360 guinea pigs died of intercurrent disease

The non-vaccinated controls died from two to seven months after challenge (see Appendix Table 1) The survival time was slightly shorter for the 3-month-old males as compared with the 7 and 11-month-old animals but the difference is not statistically significant (see further below) This is in agreement with some previous studies (*Duca* 1948, *Signorini & Benelli* 1955 and *Harasawa et al* 1956) If, therefore, a relationship between survival time and vaccination period is found among vaccinated animals this can reasonably be ascribed to a change in the degree of acquired resistance during the vaccination period rather than to a change in natural resistance with age

The survival times of the 3 month-old non-vaccinated male controls are also slightly shorter than those of the 4-month-old female controls

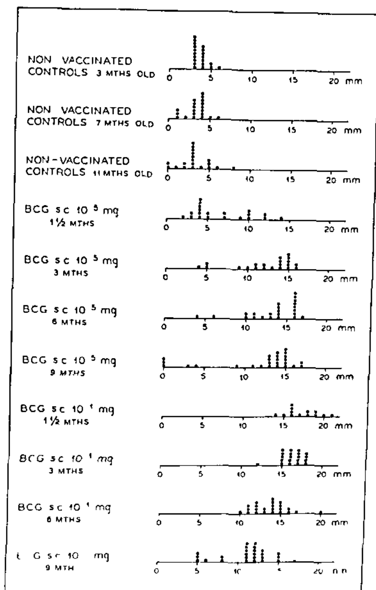
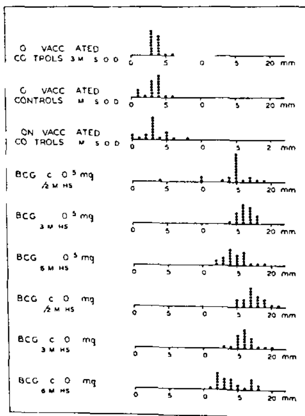



*Fig 3*

Distribution of tuberculin reactions (erythema in mm) to 250 TU of non vaccinated guinea pigs and guinea pigs vaccinated subcutaneously with $10^{-5}$ mg or $10^{-1}$ mg BCG vaccine at specified periods before testing

mg of BCG vaccine as compared to *subcutaneous* vaccination with the same dose When a larger dose of vaccine is used ($10^{-1}$ mg) the tuberculin sensitivity develops more quickly and begins to wane at an earlier stage than with the small dose In some of the animals no tuberculin sensitivity could be demonstrated after subcutaneous vaccination with $10^{-5}$ mg, regardless of the length of the vaccination period On the other hand, all of the animals vaccinated intracutaneously with the same dose were sensitive to tuberculin three months after vaccination




*F g 4*

Distr I tion of t bercul n react ns (ervthema in mm) to 250 TU of non vacc nate l gu nea p gs an l guinea p gs vaccinated intracutaneously w th 10 mg or 10 1 mg BCC vacc ne at spec fied p riods before testing

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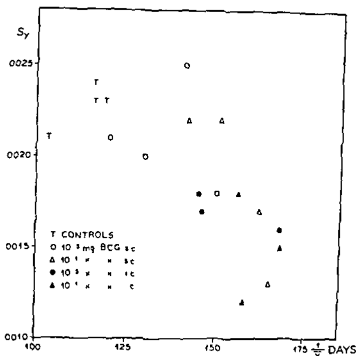



*Fig 5*

Relationship between standard deviation of reciprocal survival times and median survival times for groups of non-vaccinated guinea pigs and guinea pigs vaccinated subcutaneously or intracutaneously with BCG vaccine

Although the difference is not statistically significant, it may be a "*true*" *difference, since it has been observed in two other studies* (unpublished)

Animals vaccinated subcutaneously (Appendix Table 2) or intracutaneously (Appendix Table 3) with BCG vaccine died somewhat later, *i e* from 2½ to 12 months after challenge

A statistical evaluation of the survival times of the various groups is given in Table 4 The arithmetic means of the reciprocal survival times ($\bar{y}$), the standard deviation ($s_y$) and the median survival time in days ($\frac{1}{\bar{y}}$) are shown for each group

In Fig 5 the standard deviations are plotted against the median survival times The standard deviation decreases with increasing median survival time, a result similar to the one found in an earlier paper (*Jespersen et al* 1962)

The four control groups do not differ significantly from each other (see Table 4) The reciprocal survival time varies on an average of about $\bar{y} = 0\ 0088$, with the standard deviation $s_y = 0\ 0023$ The standard deviations for the groups vaccinated intracutaneously are all significantly lower than for the control groups The same is true for the subcutaneously vaccinated groups where the median survival time exceeded 145 days, excepting the group vaccinated with $10^{-1}$ mg of BCG 9 months before challenge

TABLE 4

*Median Survival Time (in Days) after Challenge with Virulent Tubercle Bacilli of Non Vaccinated Guinea Pigs or Guinea Pigs Vaccinated with BCG Vaccine*

| Group | | Subcutaneous vaccination | | | | Intracutaneous vaccination | | | |
|---|---|---|---|---|---|---|---|---|---|
| Vaccine dose | Vaccination period (months) | No of animals | Arithmetic mean of reciprocal survival times $\bar{y}$ | Standard deviation $s_y$ | Median survival time (in days) $\frac{1}{\bar{y}}$ | No of animals | Arithmetic mean of reciprocal survival times $\bar{y}$ | Standard deviation $s_y$ | Median survival time (in days) $\frac{1}{\bar{y}}$ |
| $10^{5}$ mg BCG | 1½ | 19 | 0.0083 | 0.0021 | 120 | 21 | 0.0068*** | 0.0017 | 146 |
| | 3 | 21 | 0.0077 | 0.0020 | 130 | 21 | 0.0069*** | 0.0018 | 145 |
| | 6 | 23 | 0.0067*** | 0.0018 | 150 | 25 | 0.0060*** | 0.0016 | 168 |
| | 9 | 25 | 0.0071** | 0.0025 | 141 | | | | |
| $10^{3}$ mg BCG | 1½ | 22 | 0.0061*** | 0.0013 | 165 | 22 | 0.0059*** | 0.0015 | 168 |
| | 3 | 21 | 0.0062*** | 0.0017 | 162 | 21 | 0.0063*** | 0.0012 | 158 |
| | 6 | 23 | 0.0070** | 0.0022 | 142 | 25 | 0.0064*** | 0.0018 | 156 |
| | 9 | 26 | 0.0066*** | 0.0022 | 151 | — | | | |
| Non-vaccinated controls | | | | | | | | | |
| Males 3 months old | | 20 | 0.0097 | 0.0021 | 103 | | | | |
| 7 " " | | 20 | 0.0084 | 0.0023 | 119 | | | | |
| 11 " " | | 20 | 0.0085 | 0.0023 | 117 | | | | |
| Females approx 4 mths old | | 20 | 0.0085 | 0.0024 | 118 | | | | |

** and *** denote that the deviation from the control groups is significant, $1\% > P > 0.1\%$ and $0.1\% > P$ respectively

TABLE 5

*Median Survival Time (in Days) after Challenge with Virulent Tubercle Bacilli of Groups of Non Vaccinated Guinea Pigs and Guinea Pigs Vacinated with BCG Vaccine Specified According to Size of Tuberculin Reaction to 250 TU at Time of Challenge*

| Group | | | Tuberculin reaction (TR) in mm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | TR < 6 | | | | 6 < TR | | | |
| Vaccination route | Vaccination dose | Vaccination period months | No of animals | Arithmetic mean of reciprocal survival times $\bar{v}$ | Standard deviation $s$ | Median survival time (in days) $\frac{1}{\bar{v}}$ | No of animals | Arithmetic mean of reciprocal survival times $\bar{v}$ | Standard deviation $s$ | Median survival time (in days) $\frac{1}{\bar{v}}$ |
| Non vaccinated controls | 3 months old | | 20 | 0.0097 | 0.0021 | 103 | 0 | – | | – |
| | 7 " | | 20 | 0.0084 | 0.0023 | 119 | 0 | – | | – |
| | 11 | | 19 | 0.0085 | 0.0024 | 118 | 0 | – | | – |
| Subcutaneous vaccination | $10^{-5}$ mg | 1½ | 10 | 0.0087 | | 115 | 9 | 0.0077 | 0.0022 | 130 |
| | | 3 | 3 | 0.0076 | | 132 | 18 | 0.0077 | 0.0018 | 130 |
| | | 6 | 2 | 0.0107 | | 93 | 21 | 0.0063 | 0.0013 | 159 |
| | | 9 | 5 | 0.0099 | | 101 | 20 | 0.0064 | 0.0016 | 156 |
| | $10^{-1}$ mg | 1½ | 0 | | | – | 22 | 0.0060 | 0.0013 | 167 |
| | | 3 | 0 | | | | 21 | 0.0061 | 0.0017 | 164 |
| | | 6 | 0 | | | – | 23 | 0.0070 | 0.0022 | 143 |
| | | 9 | 4 | 0.0087 | | 115 | 22 | 0.0062 | 0.0021 | 161 |
| Intracutaneous vaccination | $10^{-5}$ mg | 1½ | 1 | 0.0089 | | 112 | 20 | 0.0067 | 0.0017 | 149 |
| | | 3 | 0 | | | – | 21 | 0.0069 | 0.0018 | 145 |
| | | 6 | 0 | – | | – | 24 | 0.0059 | 0.0016 | 169 |
| | $10^{-1}$ mg | 1½ | 0 | | | – | 22 | 0.0059 | 0.0015 | 169 |
| | | 3 | 0 | | | – | 21 | 0.0063 | 0.0012 | 159 |
| | | 6 | 0 | | | – | 25 | 0.0064 | 0.0018 | 156 |

Significant deviations in median survival times of vaccinated groups as compared to control groups are marked in Table 4. All groups of vaccinated animals, except the two vaccinated subcutaneously with 10 mg of BCG 1½ and 3 months prior to challenge, deviate significantly from the control groups. Except for these two groups, there are no significant difference in the survival times of the vaccinated animals. For these two groups taken together, the median survival time is significantly shorter than the median survival times of the groups vaccinated subcutaneously with $10^{-2}$ mg of BCG 6 and 9 months before challenge. In contrast to this the median survival time is shorter 6 and 9 months after subcutaneous vaccination with $10^{-1}$ mg of BCG than 1½ and 3 months after vaccination. The difference is not significant as regards the median survival time, but the standard deviation is significantly greater in the groups with the long vaccination time, which might be due to a decrease in resistance in some of the animals.

*Relationship between tuberculin sensitivity and survival time*

Distribution of the groups according to tuberculin reactions below and above 6 mm (250 TU) (Table 5) shows that the survival times of the animals with small reactions generally are somewhat shorter. There is a significant difference in survival times for the groups vaccinated with 10 mg of BCG nine months prior to challenge, even though the value $s_x = 0.0023$* is used. However, as regards the $10^{-1}$ mg group the deviation is just on the borderline of significance.

A relationship between the tuberculin sensitivity and the survival time for animals within the two groups can be demonstrated in two instances. This applies to the group vaccinated subcutaneously with 10 mg of BCG six months prior to challenge (TR > 6 mm) and somewhat surprisingly to the control group (TR ≦ 6 mm) where there is a weak positive correlation (statistical analysis not shown).

The median survival time of animals with TR > 6 mm vaccinated subcutaneously with $10^{-2}$ mg of BCG 1½ and 3 months before challenge (130 days) is significantly shorter than that of guinea pigs with TR > 6 mm vaccinated at the same time with $10^{-1}$ mg of BCG (167 days and 164 days).

*Persistence of viable BCG bacteria in the vaccinated animals*

The results of culture from organs of the vaccinated animals killed at the time of challenge are shown in Table 6 together with the tuber-

* $10^{-2}$ mg BCG $\bar{y}$ (diff.) = 0.0099 − 0.0064 = 0.0035

$$SE = 0.0023\sqrt{\frac{1}{5}+\frac{1}{20}} = 0.0012$$

$10^{-1}$ mg BCG $\bar{y}$ (diff.) = 0.0087 − 0.0062 = 0.0025

$$SE = 0.0023\sqrt{\frac{1}{4}+\frac{1}{22}} = 0.0013$$

TABLE 6

*Isolation of BCG Bacteria from Vaccinated Animals*

| Group | | Animal no | Subcutaneous vacc | | Intracutaneous vaccination | |
|---|---|---|---|---|---|---|
| Vaccine dose | Vaccination period months | | Culture of lymph nodes (viable units per tube) | Tuberculin reaction to 250 TU (in mm) | Culture of lymph nodes (viable units per tube) | Tuberculin reaction to 250 TU (in mm) |
| | 1½ | 1 | 0 | 2 6 | 0 | 13 4 |
| | | 2 | 0 | 3 9 | 0 | 12 4 |
| | 3 | 1 | 0 | 12 5 | 0 | 15 6 |
| | | 2 | 2 | 15 4 | 2 | 15 5 |
| $10^{5}$ mg BCG | 6 | 1 | 20 | 15 6 | not examined | 14 3 |
| | | 2 | 29 | 13 6 | 0 | 14 9 |
| | 9 | 1 | 0 | 3 7 | – | – |
| | | 2 | 3 | 12 6 | – | – |
| | | 3 | 1 | 14 2 | – | – |
| | | 4 | 0 | 7 5 | – | – |
| | 1½ | 1 | 5 | 16 6 | 0 | 16 0 |
| | | 2 | 6 | 17 4 | 0 | 16 1 |
| | 3 | 1 | 1 | 17 4 | 0 | 17 0 |
| | | 2 | 0 | 12 8 | 0 | 16 3 |
| $10^{1}$ mg BCG | 6 | 1 | 0 | 12 4 | 0 | 14 1 |
| | | 2 | 0 | 14 9 | 0 | 11 0 |
| | 9 | 1 | 0 | 10 9 | – | – |
| | | 2 | 0* | 10 1 | – | – |
| | | 3 | 0 | 13 1 | – | – |
| | | 4 | 0 | 4 4 | – | – |

Liver Culture negative in all animals
* Culture from spleen positive, all other animals negative

culin reactions to 250 TU BCG bacteria could be cultured from nine out of twenty animals vaccinated subcutaneously and from one out of twelve animals vaccinated intracutaneously

Culture from the lymph nodes of some of the animals was positive three, six, and nine months after subcutaneous vaccination with $10^{5}$ mg and 1½ and 3 months after subcutaneous vaccination with $10^{1}$ mg It is striking that the two guinea pigs (vaccinated nine months previously) from which BCG bacteria were cultured had much stronger tuberculin reactions (12 6 and 14 2 mm, see Table 6) than the two guinea pigs from the same group with negative cultures (3 7 and 7 5 mm, see Table 6) In one case, culture from the spleen was positive However, cultures from the spleens of quite a few of the animals were contaminated and therefore inconclusive No tubercle bacilli could be cultured from the livers of the animals

BCG bacteria were isolated from only one of the animals vaccinated intracutaneously three months previously with $10^{5}$ mg of BCG vaccine The localization of the vaccination injections might have been a contributory factor in the negative culture results for the animals vaccinated intracutaneously However, it should also be noted that the difference in the frequency with which BCG bacteria were isolated from

animals vaccinated subcutaneously and intracutaneously is not statistically significant

The histological examination did not reveal any specific changes in any of the animals and microscopy (10 minutes' examination of each section) showed no tubercle bacilli

## DISCUSSION

*Acquired resistance* As already mentioned the main object of the present study was to ascertain whether a small dose of living BCG vaccine could evoke just as strong and durable a protection against tuberculosis in guinea pigs as a large dose

It is difficult to draw any definite conclusion from previous works on this subject since the experimental conditions have varied from study to study In the study by the U S Public Service Tuberculosis Program (1955a) with a vaccination period of five weeks and intracutaneous vaccination of large groups of animals the degree of resistance acquired by guinea pigs was found to increase systematically with the vaccine dose which varied from 0 00075 mg to 7 5 mg Using a vaccination period of six weeks *Cohn et al* (1958) found that out of six guinea pigs vaccinated with approximately 25 viable units of BCG vaccine by inhalation four became tuberculin positive and acquired a resistance similar to that acquired by animals which had inhaled a dose 100 times larger The two other animals vaccinated with the same small dose remained negative and had not acquired any resistance As mentioned in the introduction using a vaccination period of seven weeks and vaccine doses ranging from $10^{-6}$ mg to $10^{-1}$ mg *Jespersen* (1956) found that the degree of acquired resistance increased with the vaccine dose up to 0 0001 mg A further increase in the vaccine dose had only a slight effect Similar results were obtained in a study on red mice using the same vaccination period (*Jespersen* 1954) Using a vaccination period of six months and four different sub strains of BCG on large groups of animals *Willis et al* (1960) observed that the degree of acquired resistance increased with the vaccine dose for all four sub strains although to varying extents for the individual strains In their study the vaccine dose varied from 0 00125 mg to 0 02 mg (*Vandiviere & Willis* 1961)

In addition to the vaccination time the method of evaluating the degree of acquired resistance also varied in the different studies The survival times were used in the study by the *U S Public Health Service Tuberculosis Program* (1955a) and by *Jespersen* (1956) *Willis et al* (1960) sacrificed the animals at a certain time and judged the extent of the tuberculous infection in the individual animals *Cohn et al* (1958) killed the animals and performed quantitative culture from lungs and spleen

The challenge dose and route of infection also varied from one study to the other

TABLE 6

*Isolation of BCG Bacteria from Vaccinated Animals*

| Group | | Animal no | Subcutaneous vacc | | Intracutaneous vaccination | |
|---|---|---|---|---|---|---|
| Vaccine dose | Vaccination *period* months | | Culture of lymph nodes (*viable units* per tube) | Tuberculin reaction to 250 TU (in mm) | Culture of lymph nodes (viable units *per tube*) | Tuberculin reaction to 250 TU (*in mm*) |
| $10^{5}$ mg BCG | 1½ | 1 | 0 | 2 6 | 0 | 13 4 |
| | | 2 | 0 | 3 9 | 0 | 12 4 |
| | 3 | 1 | 0 | 12 5 | 0 | 15 6 |
| | | 2 | 2 | 15 4 | 2 | 15 5 |
| | 6 | 1 | 20 | 15 6 | not examined | 14 3 |
| | | 2 | 29 | 13 6 | 0 | 14 9 |
| | 9 | 1 | 0 | 3 7 | – | – |
| | | 2 | 3 | 12 6 | – | – |
| | | 3 | 1 | 14 2 | – | – |
| | | 4 | 0 | 7 5 | – | – |
| $10^{1}$ mg BCG | 1½ | 1 | 5 | 16 6 | 0 | 16 0 |
| | | 2 | 6 | 17 4 | 0 | 16 1 |
| | 3 | 1 | 1 | 17 4 | 0 | 17 0 |
| | | 2 | 0 | 12 8 | 0 | 16 3 |
| | 6 | 1 | 0 | 12 4 | 0 | 14 1 |
| | | 2 | 0 | 14 9 | 0 | 11 0 |
| | 9 | 1 | 0 | 10 9 | – | – |
| | | 2 | 0* | 10 1 | | |
| | | 3 | 0 | 13 1 | – | – |
| | | 4 | 0 | 4 4 | | |

Liver Culture negative in all animals

* Culture from spleen positive all other animals negative

culin reactions to 250 TU BCG bacteria could be cultured from nine *out of twenty animals vaccinated subcutaneously and from one out of* twelve animals vaccinated intracutaneously

Culture from the lymph nodes of some of the animals was positive three, six, and nine months after subcutaneous vaccination with 10 mg and 1½ and 3 months after subcutaneous vaccination with $10^{1}$ mg *It is striking that the two guinea pigs* (*vaccinated nine months previously*) from which BCG bacteria were cultured had much stronger tuberculin reactions (12 6 and 14 2 mm see Table 6) than the two guinea pigs from the same group with negative cultures (3 7 and 7 5 mm, see Table 6) In one case, culture from the spleen was positive However, cultures from the spleens of quite a few of the animals were contaminated and therefore inconclusive No tubercle bacilli could be cultured from *the livers of the animals*

BCG bacteria were isolated from only one of the animals vaccinated intracutaneously three months previously with $10^{-}$ mg of BCG vaccine The localization of the vaccination injections might have been a contributory factor in the negative culture results for the animals vaccinated intracutaneously However, *it should also be noted that the diffe*rence in the frequency with which BCG bacteria were isolated from

In the same way as the resistance the tuberculin sensitivity developed more slowly after subcutaneous vaccination with $10^{-3}$ mg than with $10^{-1}$ mg The mean size of reaction was not as high with the small dose as with the big dose partly due to some animals still being negative after vaccination with the small dose

When injected intracutaneously there was much less difference in the degree of tuberculin sensitivity induced by the two vaccine doses

As regards both vaccine doses a wane in tuberculin sensitivity could be observed with the longest vaccination periods The earliest decrease (after three months) was seen in the animals vaccinated intracutaneously with $10^{-1}$ mg of BCG and the latest (after nine months) in those vaccinated subcutaneously with $10^{-3}$ mg of BCG *i e* in the animals with the poorest vaccination Thus tuberculin sensitivity apparently begins to wane first in the groups which develop tuberculin sensitivity most rapidly In agreement with a previous study on BCG vaccinated guinea pigs (*Tolderlund et al* 1960b) the degree of sensitivity six to nine months after the vaccination was very much the same in animals vaccinated with the small and with the large dose of BCG As in the above mentioned study by *Tolderlund et al* the present work indicates that the tuberculin sensitivity of guinea pigs is maintained almost as long after vaccination with $10^{-3}$ mg vaccine as after vaccination with $10^{-1}$ mg However in humans the degree of tuberculin sensitivity seems to increase with the size of the vaccine dose and to remain at higher levels for years (*Tolderlund et al* 1960b)

*Persistence of viable BCG bacteria in the vaccinated animals* It is probable that the resistance acquired by the guinea pigs also tends to eliminate the BCG bacteria thus reducing the tuberculin sensitivity at the same stage However it is not known with certainty whether the duration of the tuberculin sensitivity and the acquired resistance in BCG vaccinated guinea pigs is conditioned by the persistence of viable BCG bacteria in the host

The present study on this aspect was carried out on a relatively small number of animals only and therefore no definite conclusions can be drawn

Culture from the regional lymph nodes was positive in about half the guinea pigs vaccinated subcutaneously Tubercle bacilli could be demonstrated up to nine months after vaccination with $10^{-3}$ mg but only up to three months after vaccination with $10^{-1}$ mg As mentioned previously it is remarkable that the two guinea pigs vaccinated nine months previously from which bacteria were cultured had much stronger tuberculin reactions than the two animals from the same group with negative cultures

Specific histological changes in liver spleen and lungs were not observed in any of the animals and microscopical examination of Ziehl Neelsen stained sections from these organs did not reveal the presence of tubercle bacilli

The present work shows that development of the acquired resistance is slightly retarded after *subcutaneous* vaccination with the small dose ($10^{-5}$ mg) as compared with the large dose ($10^{-1}$ mg). Or, resistance after subcutaneous vaccination with $10^{-5}$ mg of BCG may have developed at a slightly slower rate in this study than in a previous one (*Jespersen et al.* 1962). However, with vaccination periods of six and nine months, the median survival times of the animals vaccinated with the two doses are similar, *viz.* 150 days and 141 days for $10^{-5}$ mg, and 142 days and 151 days for $10^{-1}$ mg. At that stage apparently there is a slight decrease in the survival times for some of the animals vaccinated with the large dose. The median survival times for the animals vaccinated subcutaneously with $10^{-5}$ mg is not at any stage as long as the longest mean survival times for the other groups (165 to 168 days). This is probably due to the fact that a few animals in the groups vaccinated subcutaneously with $10^{-5}$ mg had not developed any resistance. In any event, this dose was so small that a few animals in each of the groups did not become sensitive to tuberculin.

As regards *intracutaneous* vaccination, there is no significant difference in the degree of acquired resistance for the two doses, irrespective of the vaccination period. It is remarkable that the longest median survival time was found in the groups vaccinated with the large dose 1½ months before challenge and with the small dose six months before challenge (168 days).

No significant decrease in the median survival time has been demonstrated in the present study, regardless of the dose of vaccine and the vaccination route. This is in agreement with the findings in a previous work (*Magnusson et al.* 1960), where the animals were also infected intravenously with a small dose of virulent tubercle bacilli and where, generally speaking, the same median survival times were observed two and nine months after intracutaneous vaccination of guinea pigs with 0.075 mg BCG. It would seem, therefore, that considerably longer vaccination periods than those used in the present study are required in order to reveal the relationship between the vaccine dose and the duration of acquired resistance — at least with the BCG strain used in this study.

*Tuberculin sensitivity.* The mean size of tuberculin reactions of the groups differed significantly in a number of cases, both as regards 5 TU and 250 TU. In agreement with the findings in a previous study (*Jespersen & Mackeprang* 1959), differentiation between the BCG vaccinated and the non-vaccinated control animals was more distinct using 250 TU than with 5 TU. Since in the present study the difference in mean size of reactions to the two tuberculin doses was larger for the longer than for the shorter vaccination periods, the better separation with the large dose may — in this study, at least — be due partly to the resensitizing effect of the 5 TU test in animals with waning tuberculin sensitivity (*Magnus* 1957, *Magnusson et al.* 1960 and *Tolderlund et al.* 1960 a).

In agreement with a number of previous studies (see references in *Magnusson et al* 1960) a considerable fall in tuberculin sensitivity without any significant decrease in acquired resistance has been observed in the present study

*Significance of vaccination route* In a previous study (*Ornstein & Steinbach* 1928) the tuberculin sensitivity and the degree of acquired resistance were greater after intracutaneous vaccination than after subcutaneous vaccination of animals As regards humans, intracutaneous vaccination with BCG has resulted in stronger tuberculin sensitivity than the subcutaneous method (*Someya et al* 1952 and *Edwards et al* 1953) In agreement with those studies, the present work has shown that the threshold value, *i e* the smallest effective dose, both as regards tuberculin sensitivity and acquired resistance, is higher when guinea pigs are vaccinated subcutaneously with viable BCG vaccine than when vaccinated intracutaneously


SUMMARY

Groups of female guinea pigs were vaccinated subcutaneously or intracutaneously with a fairly small ($10^{-5}$ mg) or a larger ($10^{-1}$ mg) dose of liquid BCG vaccine at various times On a certain day 1½ to 9 months after vaccination, all of the animals and the non vaccinated controls were tested intradermally with 5 tuberculin units (TU) and three days later with 250 TU Four days later a few of the animals in each group were killed and examined bacteriologically and histologically for the presence of BCG bacteria The other animals were challenged intravenously with a small dose of virulent bovine tubercle bacilli and the survival times recorded

BCG bacteria were cultured from the regional lymph nodes 3 to 9 months after subcutaneous vaccination with $10^{-5}$ mg and 1½ to 3 months after subcutaneous vaccination with $10^{-1}$ mg of BCG

All of the vaccinated animals, except some vaccinated subcutaneously with $10^{-5}$ mg developed tuberculin sensitivity This developed more quickly reached a higher level, and started to wane earlier in animals vaccinated with the large dose Tests with 250 TU gave a clearer distinction between non vaccinated and BCG vaccinated guinea pigs than 5 TU

All of the vaccinated groups except two vaccinated subcutaneously with $10^{-5}$ mg of BCG 1½ and 3 months before challenge, showed a significantly prolonged survival time as compared to the non vaccinated controls Resistance was acquired more slowly after subcutaneous vaccination with the small vaccine dose, but after six and nine months there was no difference in the degree of acquired resistance evoked by the two doses When vaccination was made intracutaneously, the small dose was as effective as the big one

*Vogelsang & Wetteland* (1960, 1961) have recently examined, on a large number of animals, the spread and persistence of BCG in the guinea pig after intracutaneous injection of 0 1 mg BCG As in the present study, BCG could be found in the regional lymph nodes for a short period only To begin with there was copious growth, but after twelve weeks the growth was sparse and occurred irregularly, to disappear completely one month later. There was scanty growth from the spleen of a few animals for the first six weeks after injection Histological examination revealed scanty and inconstant proliferation of epithelioid cells in the follicles up to the ninth week

*Relationship between tuberculin sensitivity and acquired resistance to tuberculosis* This aspect has been discussed in many publications and has given rise to widely varying evaluations Here discussion will be limited essentially to the relationship between these two properties after vaccination of guinea pigs with a small dose of viable BCG in the period during which the vaccination effect is under development This question has also been examined in other previous works (*Jespersen* 1956; *Jespersen & Mackeprang* 1959, and *Jespersen et al* 1962)

It is generally found in these experiments that either both the tuberculin sensitivity and acquired resistance of the individual animals are present, or that neither of them can be demonstrated Animals which are sensitive to tuberculin after vaccination with a small dose of BCG have a longer survival time after challenge than non-vaccinated control animals, whilst animals which have not developed sensitivity to tuberculin do not live longer than the controls On the other hand, irrespective of the vaccine dose, generally a relationship between the degree of tuberculin sensitivity and acquired resistance cannot be demonstrated, even though in some groups there are animals with small tuberculin reactions An exception has been found in the present study, where a slightly positive relationship has been demonstrated within two groups, one of which is a control group A work from the *Tuberculosis Program, U S Public Health Service* (1955b) reports the finding of a slight dependence

In two cases the tuberculin sensitivity and acquired resistance probably did not develop simultaneously A group of eight animals vaccinated with $10^{-5}$ mg of BCG eight to twelve weeks before challenge were not tuberculin positive at the time of challenge but had a significantly prolonged survival time (*Jespersen et al* 1962) This observation has been discussed previously (*Jespersen et al* 1962) In the present study guinea pigs which were sensitive to tuberculin (IR > 6 mm, 250 TU) 1½–3 months after subcutaneous vaccination with $10^{-5}$ mg of vaccine had a somewhat prolonged survival time but the increase in resistance was not statistically significant This result deviates, as far as the three-month period is concerned, from the findings in a previous experiment carried out under the same experimental conditions (*Jespersen et al* 1962)

In agreement with a number of previous studies (see references in *Magnusson et al* 1960) a considerable fall in tuberculin sensitivity without any significant decrease in acquired resistance has been observed in the present study

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SUMMARY

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The threshold dose of viable BCG vaccine was higher with subcutaneous than with intracutaneous vaccination

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The relationship between tuberculin sensitivity and acquired resistance is discussed

## APPENDIX TABLE 1

*Survival Time (in Days) after Challenge with Virulent Tubercle Bacilli and Size of Tuberculin Reactions (Erythema in mm) to 5 TU* and 250 TU at the Time of Challenge of Non Vaccinated Guinea Pigs of Varying Age

| Order of survival | Male | | | | | | | | | Female | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 months | | | 7 months | | | 11 months | | | Approx. 4 months | | |
| | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time |
| | 5 TU | 250 TU | | 5 TU | 250 TU | | 5 TU | 250 TU | | 5 TU | 250 TU | |
| 1 | 1 0 | (3 0) | 78 | 1 5 | (2 5) | 68 | 0 | (1 5) | 81 | not examined | | 72 |
| 2 | 3 0 | (4 0) | 79 | 2 5 | (3 5) | 87 | S | 3 0 | 83 | " | " | 75 |
| 3 | 4 0 | (4 5) | 81 | 3 5 | (1 0) | 91 | 0 | 2 5 | 86 | " | " | 88 |
| 4 | 1 5 | (3 5) | 82 | 3 0 | (4 5) | 96 | 1 5 | 0 | 86 | " | " | 95 |
| 5 | 2 0 | (3 0) | 83 | 3 0 | (2 5) | 100 | 0 | (3 0) | 94 | " | " | 97 |
| 6 | 0 | (2 5) | 85 | 0 | (2 5) | 104 | 2 0 | (2 5) | 101 | " | " | 101 |
| 7 | 2 5 | (3 0) | 91 | 3 5 | (2 5) | 110 | 4 0 | (4 5) | 102 | " | " | 114 |
| 8 | 3 0 | (3 0) | 97 | 0 | 0 5 | 119 | 1 0 | 4 5 | 108 | " | " | 118 |
| 9 | 0 5 | (4 0) | 99 | 3 5 | (2 0) | 121 | 2 5 | 3 0 | 118 | " | " | 119 |
| 10 | 2 5 | (2 5) | 105 | 2 5 | (4 0) | 123 | 2 5 | (0 5) | 124 | " | " | 121 |
| 11 | 2 5 | (3 0) | 109 | 1 5 | (4 0) | 126 | 2 0 | (1 5) | 124 | " | " | 132 |
| 12 | 3 0 | (3 0) | 112 | 2 5 | (4 0) | 127 | 0 | S | 125 | " | " | 138 |
| 13 | 3 0 | 4 0 | 115 | 2 5 | 0 5 | 133 | 3 0 | (8 0) | 127 | " | " | 138 |
| 14 | 3 5 | (3 0) | 117 | 2 5 | (3 0) | 143 | 2 5 | 3 0 | 147 | " | " | 143 |
| 15 | 2 0 | (3 5) | 118 | 3 5 | (3 5) | 145 | 0 | 0 | 151 | " | " | 144 |
| 16 | 2 5 | (3 0) | 123 | 3 0 | 4 0 | 145 | 0 | (2 5) | 152 | " | " | 146 |
| 17 | 1 5 | (3 5) | 129 | 2 0 | (4 0) | 151 | 1 5 | (3 0) | 157 | " | " | 153 |
| 18 | 4 0 | (5 0) | 141 | 3 5 | (5 5) | 158 | S | 5 0 | 159 | " | " | 158 |
| 19 | 3 5 | (4 0) | 143 | 1 0 | (3 5) | 171 | 0 | 4 0 | 203 | " | " | 160 |
| 20 | 3 0 | (6 0) | 169 | 2 0 | (1 0) | 211 | 1 0 | (5 5) | 210 | " | " | 201 |

Tuberculin reactions in brackets were not sharp
'S' means schratched i.e. reaction not readable
Each figure is the mean of two reactions

The threshold dose of viable BCG vaccine was higher with subcutaneous than with intracutaneous vaccination

There was no significant decrease in the degree of acquired resistance in the vaccinated animals

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| Order of survival | Male | | | | | | | | | Female | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 3 months | | | 7 months | | | 11 months | | | Approx. 4 months | | |
| | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time |
| | 5 TU | 250 TU | | 5 TU | 250 TU | | 5 TU | 250 TU | | 5 TU | 250 TU | |
| 1 | 1.0 | (3.0) | 78 | 1.5 | (2.5) | 68 | 0 | (1.5) | 81 | not examined | | 72 |
| 2 | 3.0 | (4.0) | 79 | 2.5 | (3.5) | 87 | S | 3.0 | 83 | " | " | 75 |
| 3 | 4.0 | (4.5) | 81 | 3.5 | (1.0) | 91 | 0 | 2.5 | 86 | " | | 88 |
| 4 | 1.5 | (3.5) | 82 | 3.0 | (4.5) | 96 | 1.5 | 0 | 86 | " | " | 95 |
| 5 | 2.0 | (3.0) | 83 | 3.0 | (2.5) | 100 | 0 | (3.0) | 92 | " | " | 97 |
| 6 | 0 | (2.5) | 85 | 0 | (2.5) | 104 | 2.0 | (2.5) | 101 | " | " | 101 |
| 7 | 2.5 | (3.0) | 91 | 3.5 | (2.5) | 110 | 4.0 | (4.5) | 102 | " | " | 114 |
| 8 | 3.0 | (3.0) | 97 | 0 | 0.5 | 119 | 1.0 | 4.5 | 108 | " | " | 118 |
| 9 | 0.5 | (4.0) | 99 | 3.5 | (2.0) | 121 | 2.5 | 3.0 | 118 | " | " | 119 |
| 10 | 2.5 | (2.5) | 105 | 2.5 | (4.0) | 123 | 2.5 | (0.5) | 124 | " | " | 121 |
| 11 | 2.5 | (3.0) | 109 | 1.5 | (4.0) | 126 | 2.0 | (1.5) | 124 | " | " | 132 |
| 12 | 3.0 | (3.0) | 112 | 2.5 | (4.0) | 127 | 0 | S | 125 | " | " | 139 |
| 13 | 1.8 | 4.0 | 115 | 2.5 | 0.5 | 133 | 3.0 | (8.0) | 127 | " | " | 138 |
| 14 | 3.5 | (3.0) | 117 | 2.5 | (3.0) | 143 | 2.5 | 3.0 | 147 | " | " | 143 |
| 15 | 2.0 | (3.5) | 118 | 3.5 | (3.5) | 145 | 0 | 0 | 151 | " | " | 144 |
| 16 | 2.5 | (3.0) | 123 | 3.0 | 4.0 | 145 | 0 | (2.5) | 152 | " | " | 146 |
| 17 | 3.5 | (3.5) | 129 | 2.0 | (4.0) | 151 | 1.5 | (3.0) | 157 | " | " | 153 |
| 18 | 4.0 | (5.0) | 141 | 3.5 | (5.5) | 158 | S | 5.0 | 159 | " | " | 158 |
| 19 | 3.5 | (4.0) | 141 | 1.0 | (3.5) | 171 | 0 | 4.0 | 203 | " | " | 160 |
| 20 | 3.0 | (6.0) | 169 | 2.0 | (3.0) | 211 | 1.0 | (5.5) | 210 | " | " | 201 |

Tuberculin reactions in brackets were not sharp
'S' means schratched i.e. reaction not readable
Each figure is the mean of two reactions

*Survival Time (in Days) after Challenge*
*Tuberculin Reactions (Erythema in mm) to*
*of Guinea Pigs Vaccinated*

| Order of survival | $10^{-5}$ mg BCG subcutaneously | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Vaccination | | | | | | | | | | | |
| | 1½ months | | | 3 months | | | 6 months | | | 9 months | | |
| | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time |
| | 5 TU | 250 TU | | 5 TU | 250 TU | | 5 TU | 250 TU | | 5 TU | 250 TU | |
| 1 | 3.0 | (3.5) | 17* | 9.0 | 15.0 | 87 | 2.5 | (3.5) | 87 | 3.0 | 14.0 | 16* |
| 2 | 3.0 | (5.0) | 81 | 5.0 | (5.0) | 88 | 5.0 | 5.5 | 101 | 7.0 | (4.0) | 72 |
| 3 | 1.5 | (4.0) | 86 | 4.0 | 11.0 | 102 | 3.5 | 10.0 | 113 | 1.5 | 0 | 83 |
| 4 | 3.5 | 9.5 | 88 | 5.0 | (8.5) | 103 | 13.0 | 15.5 | 127 | 4.0 | (11.0) | 90 |
| 5 | 5.5 | (2.0) | 98 | 10.5 | 15.0 | 105 | 5.0 | 13.0 | 131 | 5.5 | (2.5) | 101 |
| 6 | 3.5 | 4.0 | 102 | 3.5 | 10.0 | 112 | 4.5 | 11.5 | 131 | 4.5 | 0 | 110 |
| 7 | 3.5 | (9.5) | 104 | 11.0 | 15.5 | 116 | 11.5 | 16.0 | 136 | 4.0 | 13.0 | 115 |
| 8 | 3.0 | (7.0) | 113 | 8.5 | 15.0 | 120 | 5.0 | 9.5 | 141 | 5.0 | (13.5) | 126 |
| 9 | 6.0 | 10.0 | 117 | 6.0 | 13.5 | 125 | 11.0 | 14.5 | 144 | 5.5 | 14.0 | 130 |
| 10 | 3.0 | (4.0) | 120 | 6.5 | 13.5 | 126 | 3.5 | 11.0 | 146 | 5.0 | 13.5 | 131 |
| 11 | 9.5 | 4.0 | 125 | 8.5 | 12.5 | 128 | 8.5 | 11.0 | 147 | 6.0 | 14.5 | 137 |
| 12 | 4.5 | 8.5 | 126 | 0 | (11.0) | 128 | 9.5 | 15.5 | 148 | 9.0 | (12.5) | 148 |
| 13 | 7.0 | 13.5 | 132 | 2.0 | (4.0) | 137 | 6.5 | 14.5 | 151 | 9.0 | 14.5 | 153 |
| 14 | 3.0 | (4.5) | 135 | 10.5 | 15.0 | 145 | 10.0 | 14.5 | 157 | 3.5 | (12.5) | 158 |
| 15 | 3.0 | (4.0) | 143 | 2.5 | 12.0 | 155 | 11.0 | 14.5 | 161 | 5.0 | 12.0 | 167 |
| 16 | 3.5 | (2.5) | 147 | 8.5 | 14.0 | 161 | 8.0 | 13.0 | 172 | 11.0 | 15.0 | 168 |
| 17 | 2.5 | (3.0) | 153 | 3.0 | 11.5 | 168 | 10.0 | 15.5 | 174 | 9.0 | 17.0 | 175 |
| 18 | 5.5 | 12.0 | 166 | 11.5 | 16.0 | 176 | 10.0 | 16.5 | 183 | 9.5 | 16.5 | 178 |
| 19 | 4.5 | (12.0) | 177 | 7.5 | 14.0 | 183 | 9.0 | 14.5 | 186 | 10.0 | 15.5 | 182 |
| 20 | 4.0 | (7.0) | 228 | 8.5 | 15.0 | 218 | 9.0 | (15.5) | 202 | 4.5 | 8.5 | 187 |
| 21 | | – | § | 5.0 | (5.0) | 231 | 5.0 | (15.5) | 207 | 10.5 | 14.5 | 193 |
| 22 | | – | § | – | | § | 12.5 | 16.0 | 249 | 5.0 | 13.0 | 197 |
| 23 | | | | | | | 9.0 | 15.5 | 307 | 8.5 | (15.0) | 197 |
| 24 | | | | | | | – | – | § | 7.5 | 13.5 | 210 |
| 25 | | | | | | | | | § | 4.0 | 0 | 215 |
| 26 | | | | | | | | | | 5.0 | 14.5 | 265 |

Tuberculin reactions in brackets were not sharp
Each figure is the mean of two observations
* Animal with tuberculosis index ≦ III
§ Animal died before challenge

TABLE 2

*with Virulent Tubercle Bacilli and Size of 5 TU and 250 TU at Time of Challenge Subcutaneously with BCG Vaccine*

| 10⁻⁴ mg BCG subcutaneously | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| period | | | | | | | | | | | |
| 1½ months | | | 3 months | | | 6 months | | | 9 months | | |
| Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time |
| 5 TU | 250 TU | | 5 TU | 250 TU | | 5 TU | 250 TU | | 5 TU | 250 TU | |
| 7.5 | 20.5 | 117 | 11.0 | 16.0 | 30* | 9.0 | (13.0) | 76 | 2.0 | (4.5) | 99 |
| 8.5 | 19.0 | 126 | 10.0 | 16.5 | 95 | 11.0 | 15.5 | 102 | 3.0 | (5.5) | 103 |
| 12.0 | 16.0 | 126 | 10.0 | (17.5) | 116 | 5.0 | (11.0) | 109 | 5.0 | 12.0 | 116 |
| 8.5 | 16.0 | 130 | 10.0 | 15.5 | 131 | 6.0 | 14.5 | 112 | 0 | (10.5) | 117 |
| 10.5 | 18.5 | 132 | 10.0 | 15.0 | 132 | 8.0 | 11.5 | 114 | 8.0 | (13.0) | 118 |
| 13.5 | 18.0 | 147 | 6.5 | 15.0 | 139 | 5.0 | 11.0 | 114 | 3.5 | 15.0 | 121 |
| 5.5 | 15.0 | 148 | 10.5 | (15.5) | 140 | 4.5 | 10.0 | 125 | 4.0 | (5.0) | 121 |
| 10.0 | 15.5 | 151 | 9.0 | 15.0 | 143 | 10.5 | 16.5 | 125 | 7.5 | 11.0 | 122 |
| 11.5 | 17.5 | 160 | 8.5 | 15.0 | 151 | 8.5 | 13.5 | 128 | 0 | (10.5) | 123 |
| 9.5 | 20.0 | 161 | 6.0 | (12.0) | 151 | 6.0 | 14.5 | 135 | 5.0 | 13.0 | 124 |
| 6.0 | (16.0) | 163 | 9.0 | (17.0) | 152 | 8.0 | 13.0 | 147 | 4.0 | (13.0) | 128 |
| 8.5 | (13.5) | 166 | 10.5 | 15.0 | 157 | 2.5 | (11.0) | 150 | 7.5 | 11.0 | 128 |
| 8.5 | 16.5 | 188 | 9.5 | 16.5 | 162 | 4.0 | 12.0 | 151 | 8.0 | 15.0 | 138 |
| 11.5 | 17.5 | 189 | 8.5 | 16.0 | 170 | 5.5 | 14.0 | 164 | 5.5 | (5.0) | 144 |
| 9.0 | 14.0 | 190 | 12.0 | 15.5 | 184 | 5.0 | 13.5 | 164 | 5.5 | 12.0 | 147 |
| 11.0 | 16.5 | 192 | 11.0 | 17.5 | 204 | 7.0 | 12.0 | 165 | 3.5 | 12.5 | 147 |
| 11.0 | 16.5 | 199 | 10.0 | 17.5 | 215 | 4.5 | (10.5) | 165 | 5.5 | 10.5 | 156 |
| 12.0 | 17.5 | 204 | 10.0 | 15.0 | 217 | 10.5 | (15.0) | 174 | 3.5 | 15.0 | 175 |
| 9.0 | 16.5 | 204 | 12.5 | 16.5 | 217 | 12.5 | 15.5 | 188 | 3.5 | (9.0) | 178 |
| 6.5 | 17.0 | 225 | 12.0 | 17.5 | 239 | 12.5 | 19.5 | 210 | 4.5 | 12.0 | 207 |
| 10.5 | 15.5 | 230 | 6.5 | 15.0 | 247 | 4.0 | 13.5 | 215 | 5.0 | 11.5 | 241 |
| 10.5 | 18.0 | 242 | 9.5 | (17.0) | 261 | 6.5 | (14.0) | 273 | 2.5 | 9.0 | 265 |
| | | | | | | 9.0 | (15.0) | 320 | 3.5 | (11.0) | 302 |
| | | | | | | | – | § | 3.0 | 12.0 | 318 |
| | | | | | | – | – | § | 7.0 | 12.0 | 304 |
| | | | | | | | | | 12.0 | 16.5 | 356 |

APPEND

*Survival Time (in Days) after Challen*
*Tuberculin Reactions (Erythema in mm)*
*of Guinea Pigs Vaccina*

| Order of survival | 10⁻⁵ mg BCG intracutaneously | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Vaccinat | | | | | | | | |
| | 1½ months | | | 3 months | | | 6 months | | |
| | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time |
| | 5 TU | 2.0 TU | | 5 TU | 2.0 TU | | 5 TU | 2.0 TU | |
| 1 | 4 5 | 15 0 | 104 | 10 0 | (14 5) | 86 | 10 0 | (14 0) | 108 |
| 2 | 8 5 | 14 5 | 109 | 12 5 | 18 0 | 90 | 4 5 | 14 0 | 114 |
| 3 | 3 0 | (3 5) | 112 | 12 0 | 16 0 | 126 | 11 0 | 19 0 | 129 |
| 4 | 7 0 | 14 5 | 118 | 12 5 | 17 0 | 129 | 10 5 | 14 0 | 131 |
| 5 | 6 0 | 13 5 | 119 | 10 5 | 15 5 | 130 | 10 0 | 13 0 | 139 |
| 6 | 8 0 | 14 5 | 122 | 11 0 | 14 5 | 130 | 5 5 | 12 0 | 139 |
| 7 | 11 0 | 17 5 | 128 | 10 0 | 14 5 | 136 | 8 0 | (12 5) | 141 |
| 8 | 10 5 | (14 5) | 130 | 13 0 | 16 0 | 140 | 9 0 | 15 5 | 147 |
| 9 | 3 5 | 10 0 | 133 | 11 0 | 16 5 | 143 | 9 0 | 16 0 | 155 |
| 10 | 9 0 | 18 5 | 135 | 11 5 | 17 0 | 145 | 3 0 | S | 156 |
| 11 | 9 0 | 15 5 | 153 | 11 0 | 17 5 | 147 | 4 0 | 12 5 | 156 |
| 12 | 7 5 | 6 5 | 159 | 12 5 | 16 0 | 155 | 6 0 | 13 5 | 157 |
| 13 | 11 5 | 4 0 | 160 | 12 0 | 16 0 | 156 | 10 0 | 12 0 | 158 |
| 14 | 7 0 | 5 0 | 162 | 9 5 | (17 0) | 158 | 8 0 | 15 0 | 174 |
| 15 | 9 0 | 4 5 | 165 | 8 0 | 14 0 | 161 | S | (16 0) | 180 |
| 16 | 12 0 | (15 0) | 172 | 10 0 | 15 0 | 163 | 10 0 | 17 5 | 186 |
| 17 | 5 0 | 16 5 | 172 | 14 5 | 17 0 | 185 | 10 0 | 15 5 | 195 |
| 18 | 5 5 | 15 0 | 188 | 11 0 | 16 0 | 189 | 6 0 | 14 0 | 209 |
| 19 | 5 5 | 13 0 | 222 | 11 0 | (17 5) | 199 | 10 0 | 15 0 | 218 |
| 20 | 7 0 | 15 0 | 232 | 12 5 | 17 0 | 216 | 9 0 | 11 5 | 218 |
| 21 | 5 0 | 10 0 | 306 | 12 0 | 16 5 | 240 | 6 0 | 16 5 | 220 |
| 22 | | | * | – | – | * | 11 5 | 16 0 | 237 |
| 23 | | | | | | | 11 0 | 15 0 | 241 |
| 24 | | | | | | | 7 5 | 14 0 | 245 |
| 25 | | | | | | | 10 5 | 14 5 | 251 |

Tuberculin reactions in brackets were not sharp
S' = schratched *i e* reaction not readable
Each figure is the mean of two observations
* Animal died before challenge
§ Animal killed one year after challenge Tuberculosis index on autopsy = IV

TABLE 3

*with Virulent Tubercle Bacilli and Size of 5 TU and 250 TU at Time of Challenge Intracutaneously with BCG Vaccine*

| $10^{-4}$ mg BCG intracutaneously | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Period | | | | | | | | |
| 1½ months | | | 3 months | | | 6 months | | |
| Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time | Tuberculin reactions | | Survival time |
| 5 TL | 250 TL | | 5 TL | 250 TL | | 5 TL | 250 TL | |
| 14 0 | 17 0 | 119 | 9 0 | 16 0 | 118 | 5 5 | (13 5) | 99 |
| 10 0 | 16 5 | 122 | 11 5 | (18 5) | 129 | 1 0 | 12 0 | 101 |
| 13 5 | 17 0 | 122 | 9 0 | 15 0 | 130 | 2 5 | 10 5 | 104 |
| 9 5 | (18 5) | 138 | 8 5 | 13 5 | 134 | 9 0 | 17.5 | 121 |
| 11 0 | 16 5 | 144 | 11 5 | 15 5 | 138 | 3 0 | 13 5 | 126 |
| 12 0 | 17 0 | 145 | 9 0 | 16 0 | 139 | 12 0 | 17 0 | 131 |
| 12 0 | 14 5 | 146 | 12 0 | 17 5 | 144 | 7 0 | 17 5 | 135 |
| 13 0 | 16 5 | 149 | 10 0 | 15 0 | 153 | 6 0 | 12 0 | 141 |
| 10 5 | 16 0 | 153 | 8 5 | 16 0 | 155 | 9 0 | 16 5 | 143 |
| 8 0 | 20 5 | 162 | 12 5 | 16 5 | 156 | 8 5 | 12 0 | 153 |
| 12 0 | 19 0 | 164 | 13 5 | 16 0 | 159 | 10 0 | 11 5 | 157 |
| 10 0 | 19 5 | 173 | 11 0 | 19 5 | 160 | 11 0 | 16 0 | 161 |
| 14 5 | 18 5 | 175 | 7 0 | 13 0 | 161 | 6 0 | 12 0 | 167 |
| 11 0 | (17 5) | 180 | 9 0 | 15 0 | 168 | 0 | 12 5 | 170 |
| 11 0 | 16 0 | 181 | 9 5 | 16 5 | 172 | 6 5 | 17 0 | 172 |
| 11 5 | 17 5 | 187 | 12 0 | 14 5 | 173 | 1 5 | 12 5 | 172 |
| 12 5 | 16 5 | 193 | 12 5 | 17 0 | 176 | 7 0 | 17.5 | 174 |
| 11 5 | 17 5 | 203 | 9 5 | 15 0 | 176 | 9 5 | 13 0 | 177 |
| 11 5 | 15 0 | 212 | 11 0 | 15 5 | 203 | 5 0 | 14 5 | 179 |
| 12 0 | 16 0 | 281 | 10 5 | 17 0 | 240 | 6 0 | 13.5 | 186 |
| 9 5 | 14 5 | 285 | 11.5 | 15 5 | 285 | 9 0 | 17 0 | 186 |
| *12 0* | *17 5* | *343* | | | —* | 10 0 | 13 0 | 221 |
| | | | | | | 4 5 | 14 5 | 247 |
| | | | | | | 3 5 | 13 5 | 264 |
| | | | | | | 3 0 | 12 0 | 365§ |

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FROM STATENS SERUMINSTITUT, COPENHAGEN, DENMARK
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# THE USE OF FILTER PAPER DISCS FOR AGAR GEL PRECIPITATION

By

SONJA SCHEBEL

Received 16 iii 6?

During recent years clinical and biological investigations have utilized with increasing frequency serological tests in which antigen and antibody diffuse toward each other through agar gel to react with each other forming precipitate bands whose situation depends upon the rate of diffusion and the concentration of the reacting substances.

Many forms of agar gel precipitation tests have been described since they were introduced by *Oudin* (7, 8) and *Ouchterlony* (5, 6). Later various forms of micromethods have been developed, *e.g.*, the one described by *Wadsworth* (9).

However, the macromethods as well as micromethods presuppose technical equipment in the form of various moulds and cutters which are not so easy to procure for all laboratories. Moreover, the current methods involve certain technical difficulties. An attempt was made therefore to simplify the method. Instead of wells as reservoir, discs of filter paper were used as reservoir. These discs were of the same type as those used in bacterial sensitivity tests.

The special problems associated with diffusion from discs of filter paper into the surrounding and underlying agar have previously been studied by *Frolund Thomsen* and reported in his paper on bacterial sensitivity tests (4). The present study was therefore concerned [illegible]

[illegible] with the same good result as agar gel precipitation by the usual *Ouchterlony* method.

*Elek* (2, 3) has previously tried using filter paper as reservoir for antigen and antibody respectively. He used strips of filter paper. After having been immersed in antibody, the strips were embedded in the agar while the latter was still liquid. On the gelled agar he then placed strips of filter paper soaked in antigen at right angles on the embedded ones. *Elek* also used a modification of this method. Instead of placing antigen imbibed paper on top of the agar he inoculated by a loop in the form of a [illegible] across the plate the organisms whose

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*Fig 1*

12 d scs of filter paper in 2 rows The discs in the first row contain antibody Read ng from the left undilute l ½ ¼ etc All the discs in second row contain the same amount of ant gen (thyr globulin 0 4 per cent)

plate is t be stained it is advisable to wash out unprecipitated protein first by p uring it over with phys logical saline and leaving it to stand for 24 hours This lo sens most f the discs f filter paper and the remainder are easy to remove with p ncers The plate is left f r yet another 24 hours at room temperature with distilled water Then it is drie l at r om tempertaure for approx 24 hours stained for 7 m nutes with Am do black and rinsed 4 times for 5 minutes with a solution of methanol After drying the plate may be stored During this drying and staining process the agar may lo sen a bit at the edges but this may be avoided by adding 1 per cent glycerin to the rinses in order to prevent total drying of the agar

## RESULTS

To illustrate what is obtainable by the above mentioned method, it was tested against a simple antigen antibody system which consists of thyroglobulin and sera containing antithyroglobulin This test was carried out as double diffusion in one dimension and the discs of filter paper were placed in 2 rows of 6 discs (cf Fig 1) The antigen i e the thyroglobulin was used in a 0 4 per cent solution and placed in the second row In the first there was a dilution series of the serum to be investigated placed so as to have the undiluted serum in the top left hand corner and the most diluted serum to the right As evident from the illustration the precipitation bands were of the same situa tion and the same app earance as in the original Ouchterlony technique The time which elapsed before a precipitation band appeared differed for the different antisera With the present antigen antibody system bands appeared at the end of from 24 hours to 14 days

By way of control the same experiment was carried out with wells *cut into the agar These wells were of the* same size as the discs of filter paper and placed at the same mutual distance This showed that the bands appeared on the same day and in the same numbers as with the filter paper disc method This control experiment was carried out using a strong as well as a weak antibody, since it might be imagined that with a weak antibody it would be easier to spot a difference if some antibody should be retained in the disc of filter paper But this did not occur with the quantities used in the antigen antibody system con cerned

toxin-producing properties he wanted to investigate With both methods, precipitation bands appeared in the angle between antigen and antibody Since it was difficult to obtain sufficiently high concentrations of the reacting substances in the filter paper, Elek tried to increase the concentration by drying in successive amounts in the filter paper before embedding it However, this method has never been generally adopted

The disadvantage of Elek's method was the slight absorption ability of the filter paper It seemed reasonable, therefore, to use instead discs of filter paper which are thicker and thus able to absorb a greater quantity in a smaller area

After a number of preliminary experiments, the author succeeded in arriving at the following method which appears to be applicable where otherwise the conventional Ouchterlony technique would be used

## MATERIAL

Petri dishes of plastic with a diameter of 8 75 cm

Difco special agar 1 per cent in physiological saline with 1 per cent sodium azide

Disc A round filter No 2247 of 6 mm from Messrs Carl Schleicher & Schull Dassel Germany

Antigen Thyroglobulin prepared by the method of *Derrien et al* (1) dissolved in buffered saline pH 7 38

Antibody Human sera containing antibody to thyroglobulin stored until use at —20° C

Dye A saturated solution of Amido black 10 B in the rinse mentioned below filtered and diluted with 4 volumes of rinse

Rinse Methanol 4700 ml glacial acetic acid 1050 ml water 4700 ml

## METHOD

Petri dishes were poured with 3 ml of molten agar solution With Petri dishes of the above mentioned diameter 3 ml has proved to be the smallest quantity which can just cover the entire bottom of the dish by a layer of approx ½ mm Since it is important that this layer be as uniform as possible it is necessary to use dishes with a flat inner surface and to prepare the agar plates in a controlled horizontal position

After the agar had gelled the discs of filter paper were placed on the agar in the pattern most suitable for the purpose of the test Accuracy in the distance between the individual discs was obtained by placing underneath the dish a piece of paper on which the pattern was drawn The most suitable distance between the discs in [illegible] — was found to be 6 mm measured from peri[illegible] ed on the agar with a curved ocular forceps [illegible] from the agar very quickly it is necessary [illegible] y as soon as possible after placing the discs [illegible] antibody employed was 0 02 ml this being the maximum amount which the disc can absorb without anything leaking out Such leakage should preferably be avoided since it alters the distance that the substance is to diffuse In order to prevent drying the Petri dish was then placed in a moist [illegible] ° – ° C As moist chambers one can use [illegible] with a little water in the bottom [illegible] bands between two sources of diffusion were read daily [illegible] against a black background and with illumination from the side Daily readings were made since not only the appearance of the precipitates but also changes in the pattern are of importance If desired the finished plate with the precipitation lines may be photographed or dried and stained If the

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# AN APPROACH TO TRANSFORMATION STUDIES IN MORAXELLA

*By*

KJELL BØVRE and S. D. HENRIKSEN

Received 28.iii.62

Genetic transformation of bacteria is considered well suited for the elucidation of general biochemical principles which implicate nucleic acids in genetic mechanisms (6, 13). Transformation also seems to offer the possibility of studying the genetic relationship between bacterial strains as the degree of compatibility of the DNA's of recipient and donor cells may be interpreted to reflect their genetic homology (14, 15 a.o.).

When relationships between strains, species and genera are investigated by means of transformation, a quantitative or semiquantitative procedure is most often necessary. However, an exact quantitation is no guarantee that a given chart of transformation ratios is representative, especially when only one strain of each species is included. It should be kept in mind that special affinities of a single strain might possibly cause confusion, until it has been proved that the strain is correctly named and that other strains of the same species behave similarly.

The series of events leading to the formation of transformants (1, 7, 12) may be markedly influenced by variations in test conditions. Even when different DNA's act simultaneously on samples from the same recipient culture, there may be factors which are not sufficiently under control for a quantitative measure. For instance, the different DNA's may be operating at different degrees of saturation. As pitfalls are numerous, a quantitative transformation system should be built up step by step, with detailed studies of single factors. Before such studies can be accomplished, however, there is need for a preliminary procedure which yields transformants in at least part of the system. It is our intention to give a description of such a simple fundament for transformation studies in genus Moraxella, with streptomycin resistance as the genetic marker. It resembles principally the one used by *Catlin* in transformation of Neisseriae (4, 5).

## MATERIAL AND METHODS

*Strains used.* Moraxella nonliquefaciens 826, 2770, 4455 and 4456 were all isolated from nose cultures of outpatients. Moraxella bovis 10900 was received from the

The method has been successfully used also for precipitation studies of hepatic protein against the corresponding antisera Also for comparative studies of antigens and antibodies it has proved applicable, as it is very easy to arrange the discs of filter paper in a way suitable for this purpose This gives the usual information regarding the identity or non-identity of the substances depending on the crossing, spur formation, or interference of the precipitation bands

## DISCUSSION

For testing a simple antigen-antibody system the method has proved equally applicable as the classical method Its great advantage is that very little equipment is needed, for instance no cutters or moulders Therefore, it is very easy to change the arrangement of the reservoirs, so that at all times it is possible to use the most suitable pattern Furthermore, it possesses many of the advantages of microtechnique requiring only very small quantities of antigen and antibody, *i e* 0 02 ml The thin layer of agar reduces the cloudiness of the gel, and this diminution of the third dimension facilitates the detection of faint, densely placed bands It also facilitates the microscopic study, photography, and staining of the bands The method is so simple that it is applicable with ease in the quantitative study of antigens or antisera

## SUMMARY

The author describes a simple method for performing agar gel precipitation using discs of filter paper as reservoirs for antigen and antibody This method has proved easy and practical and appears to be up to the conventional Ouchterlony method in the investigation of thyroglobulin antibodies and other antigen-antibody systems

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more than 20 experiments with extensive variations in culture age composition of media dilution and oxygen tension before DNA exposure as well as variations in the transformation procedure proper None of the recipient cells was able to grow in the presence of 1 µg streptomycin per ml applied in the same way as the 500 fold greater quantity of streptomycin in the performance of transformant selection, i.e. from the under surface of blood agar inoculated 6 h earlier and incubated at 32° C.

*Transformation procedure* 1 ml of recipient culture was added to 1 ml of brain

spread ng annroor ato

into another Petri dish of the same size, containing beforehand a layer of 4 ml agar with 3 mg streptomycin per ml After close contact had been obtained between the two layers of agar by simple manoeuvres this would give a total of 500 µg streptomycin per ml after diffusion had taken place The incubation was continued for 2 3 days with plates bottom up in the same athmosphere Counts were then made of transformants Number of exposed bacteria could be counted earlier, as the transformants generally had a pronounced lag period

Transformant colonies in a number of 10 to 20 from each transformation were controlled by cultivation on brain heart infusion agar containing 500 µg streptomycin per ml and on medium without streptomycin All suspected transformant colonies tested were streptomycin resistant On no occasion growth of Moraxella occurred on streptomycin plates after exposure of sensitive bacteria to DNA

agar handled carefully The accuracy of counts seemed considerable, as several multiple series of readings seldom gave values differing by more than 10 per cent

TABLE 1

*Exploratory Transformation Tests with Moraxella Strains*

| Recipient Strain | Exposed Cells per ml | Transformants per ml with DNA Extracted from Strain | | | |
|---|---|---|---|---|---|
| | | 826 | 7784 | 9425 | 7911 |
| M nonliquefaciens 826 | 1.5 × 10⁸ | < 10 | < 10 | < 10 | < 10 |
| M "lacunata" 7784 (nonliquefaciens)* | 6.3 × 10⁸ | 31600 | 35000 | 250 | < 10 |
| M bovis 9425 | 4 × 10⁷ | < 10 | < 10 | < 10 | < 10 |
| M liquefaciens 7911 | 3.8 × 10⁷ | < 10 | < 10 | < 10 | < 10 |

Each of the horizontal lines represents simultaneous exp
the same
refer to t
numbers

* This strain was originally considered to be a strain of Moraxella lacunata, but biochemically and culturally it behaved like a nonliquefaciens strain

American Type Culture Collection The remaining strains were received from the National Collection of Type Cultures London These are Moraxella bovis 9425 *Moraxella liquefaciens 7911 and Moraxella "lacunata" 7784* which in our hands both biochemically and according to transformation pattern turned out to be a non liquefaciens strain All strains were studied biochemically and microscopically to ensure that they were correctly named (3 8 9, 10)

*Selection of streptomycin resistant mutants* 4 strains were subjected to selection of mutants for DNA extraction Mutants from Moraxella nonliquefaciens 826 and Moraxella "lacunata" 7784 were selected as described by *Catlin* (4) The streptomycin sensitive mother strains were cultured in brain heart infusion broth (Difco) with aeration for 18 h at 32° C A quantity of streptomycin sulphate sufficient to give 500 $\mu$g per ml was then added After further incubation without aeration surface inoculation was made on brain heart infusion agar containing the same quantity of streptomycin per ml Mutants from Moraxella liquefaciens 7911 were selected by inoculation of streptomycin agar surface with the sediment from a centrifuged 18 h old aerated broth culture without streptomycin Mutants from Moraxella bovis 9425 were isolated by inoculation of streptomycin free agar surface with the sediment from an 18 h old aerated broth culture devoid of streptomycin After growth for approximately 6 h the agar layer was lifted from the Petri dish into another Petri dish containing a thin layer of agar with streptomycin in sufficient quantity to give 500 $\mu$g per ml after diffusion through the overlying agar This method of moving inoculated agar medium with the growth side up from one Petri dish to another, is described in the section of transformation procedure

One mutant colony from each strain was selected for the following DNA extraction and transformation experiments, after control of its growth in medium containing 500 $\mu$g streptomycin per ml and in medium without streptomycin excluding streptomycin dependence

*DNA preparation* Each selected mutant was subjected to the following procedure to obtain transforming DNA solutions The surface growth of 30 blood agar plates was harvested after incubation in a humid athmosphere at 32° C for 20 h The bacteria were suspended in 70 ml of citrate buffered saline (0 14 M NaCl + 0 015 M sodium citrate pH 7 4), the standard buffer of *Zamenhof et al* (16) Granular sodium dodecyl sulphate was added to a final concentration of 3 to 4 per cent and the suspension placed in a 68° C water bath for 30 to 45 min In this way lysis seemed to be enhanced as compared with lower temperatures The relative termostability of the transforming principle has been shown by *Zamenhof et al* (16) *Avery et al* (2) have used high temperatures to inactivate deoxyribonuclease before extraction That sufficient lysis had taken place was controlled by microscopic inspection of the resulting emulsion After cooling 2 volumes of 96 per cent ethanol were slowly added while the container was vigourously rotated by hand The resulting fibrous precipitate was lifted by means of a metal strainer and excess alcohol drained off Part of the precipitate was flocculent and this part was obtained by centrifugation After draining off excess alcohol by inverting the tubes both precipitates were dissolved together in 70 ml of 1 M NaCl by mechanical stirring at 5° C for 16 to 20 h The resulting solution was then centrifuged (20000 × G 5° C 1 h) in cellulose nitrate tubes sterilized by UV irradiation The supernatants were collected and precipitated with 1 volume of 96 per cent ethanol As in the preceding alcohol precipitation two kinds of precipitate resulted the one floating coherent and glittering white the other flocculent and slowly sedimenting The precipitates were obtained and drained as described finally dissolved in standard buffer and stored at —20° C After adjusting the DNA content to 75 $\mu$g per ml by the conventional diphenylamine reaction of Dische this crude extract was used in trans [illegible] dilution in brain heart infusion broth

deoxyribonuclease (Sigma) was dissolved in a concentration of 10 mg per ml and stored in the refrigerator Immediately before use a mixture was prepared in the proportions 1 part of *deoxyribonuclease* solution to 4 parts 2 M $MgCl$ to 5 parts of 2 per cent gelatin In the assay procedure a quantity of mixture was used sufficient to give 10 $\mu$g per ml DNA solution or DNA/culture mixture

*Recipient cells* 12 h old blood agar cultures with an additional growth period of 1½ h in brain heart infusion broth were generally used in assays Incubation at 32° C in a humid atmosphere was applied As a rule no attempt was made to obtain maximal competence However Moraxella nonliquefaciens 826 was subjected to

with the reliability of counts when this method is used Even slightly reduced oxygen tension may impair growth of these organisms, a further fact in favour of surface growth The method also permits considerable prolongation of expression time, which may be of importance in future studies of phenotypic expression

The taxonomy of genus Moraxella and its relation to other genera, *e g* Neisseria, are matters under discussion (8, 9, 10, 11) Criteria for classification are few, in part due to low biochemical reactivity in common tests Transformation studies may perhaps elucidate some obscure points of relationship, when a quantitated and representative system has been built up Apart from some information concerning the applicability of transformation studies in genus Moraxella, the present paper does not answer questions concerning classification There is, however, a provisional probability that Moraxella bovis and Moraxella nonliquefaciens in terms of transformation ratios may be said to have been correctly classified as two different species of the same genus (14, 15) At present there is no other literature report on transformation of Moraxella


## SUMMARY

A tentative method for transformation studies in Moraxella is described Evidence for intraspecific and interspecific transformation is presented Provisional transformation ratios for Moraxella nonliquefaciens and Moraxella bovis may indicate their correct classification as *two* distinct species of the same group, but no definite answer as to the taxonomic validity of this observation can be given at the present time


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## RESULTS

Some of the results obtained in transformation attempts following the described method are listed in Table 1 Moraxella nonliquefaciens 826 was also negative as recipient in more than 20 other experiments where the procedure was extensively varied, including the use of 8 different homospecific DNA extracts The transformation of Moraxella "lacunata" 7784 by DNA extracts 826 and 7784 has been evident in numerous additional experiments, and the one by DNA extract from Moraxella bovis 9425 in *one more experiment following the standard* procedure In the latter experiment the yield of transformants was generally lower, but the proportions of heterospecific and isospecific to *homospecific transformation were practically the same as can be dedu*ced from Table 1 Single tests, in which DNA exposure time was prolonged to 1 h, revealed distinct evidence of transformation by DNA ex*tract 826 of 3 additional M nonliquefaciens strains 2770, 4455 and 4456* One of these strains, M nonliquefaciens 2770, was also tested in the standard procedure with 15 min exposure time and simultaneous exposure to all 4 DNA's The transformant yield was low, but approximately the same with DNA's 826 and 7784 As might be predicted, no transformants could be found at this low level of competence with DNA 9425 nor with DNA 7911

Moraxella bovis 9425 was in additional tests invariably useless as recipient, whereas M bovis 10900 repeatedly was transformed by DNA extract 9425 The strain 10900 later died before it could be treated simultaneously by different DNA extracts according to the procedure described

M liquefaciens 7911 was not transformed by homospecific DNA in any of a series of varied attempts, neither would this DNA transform other recipients in additional tests

In an early experiment essentially following the described method with 10 $\mu$g DNA per ml and 15 min exposure time, both homospecific and isospecific transformation of M "lacunata" 7784 yielded more than 3 per cent transformants, which is approximately a 6 times greater yield than listed in Table 1 It is obvious from the many preliminary experiments which have been undertaken, that competence of being transformed fluctuates when no stabilizing measure is used

## DISCUSSION AND CONCLUSION

The described fundamental method for transformation studies in Moraxella does not differ essentially from other, known, quantitative procedures, but it has some advantages which seem to make it well fit for studies on transformation It permits the direct study of transformant colonies on the surface of the medium, which is principally safer

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THE SERUM AND TOXOID DEPARTMENT, STATENS SERUMINSTITUT, COPENHAGEN

# WEEVERFISH TOXIN

## *Some Physico Chemical and Immunological Observations*

*By*

E SKEIE

Received 19 iv 62

A previous work reported an extraction method which the writer found most suitable for extracting weeverfish toxin (*Skeie* 1962a) As mentioned in that work, the toxicity of the different weeverfish toxin preparations produced by the writer varied considerably Allowing for the amounts of saline used for extraction, the toxicity of the secretion removed from the glands varied from 2,000 to 10 000 DML per ml when assayed intravenously on white mice weighing 16 to 18 grams

Comparison of the venomousness of weeverfish toxin for small animals with that of other animal toxins, e g from the common European types of viper, *Vipera aspis*, *Vipera ammodytes* and *Vipera berus*, is difficult, since the toxicity of the latter also varies (*Bieling et al* 1936) However, generally speaking, they appear to be about the same order of magnitude

Though much is known from the literature concerning the effect of weeverfish toxin on various small animals (for referencees see *Skeie* 1962b) only little information is available concerning its composition *Halstead* (1957) writes "Nothing is known of the chemistry of weever venom In 1960 *Russel & Emery*, in a work on weeverfish toxin, reported the percentage composition of what they called the toxin in a preparation produced by extraction of all the tissue in the immediate neighbourhood of the poison spines However, according to the authors the toxicity of the preparation was too weak to permit determination The figures are therefore confusing and cannot be taken [illegible]

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Comparison of the venomousness of weeverfish toxin for small animals with that of other animal toxins, e.g. from the common European types of viper, *Vipera aspis*, *Vipera ammodytes* and *Vipera berus*, is difficult, since the toxicity of the latter also varies (*Bieling et al.* 1936). However, generally speaking, they appear to be about the same order of magnitude.

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The author of the present study has extracted the venom only from the glands themselves and has tried to characterize and purify the toxin by means of various purification methods.

Financial assistance for this work has been received from *P. Carl Petersen's Foundation* and the *Danish State Research Foundation* (*Statens almindelige Videnskabsfond*).

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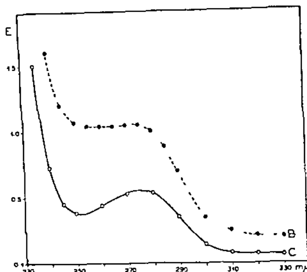



*Fig 1*

Spectrophotometry of Fj A diluted 1 20
Curve B immediately after extraction Curve C after thawing from —60° C and standing at room temperature for 14 days The abscissa shows the various wave lengths and the ordinate the extinction

has been precipitated This was confirmed by Kjeldahl analysis of the same preparation, the protein content before and after precipitation being 10 and 6 mg per ml respectively Since the toxicity was unchanged, an approximately two times purer toxin can be obtained in this way without loss of specific material

*Paper electrophoresis* was performed on the partly purified toxin preparation Fj A This was carried out in Tris buffer at 0 25 mA cm and 90 V for 18 hours After drying, the paper was stained with amido black and the result of the electrophoresis measured in a Spinco Analytrol recorder The curve obtained is shown in Fig 2

The curve revealed three main fractions – one which moved very slowly, and two moving more rapidly This is in good agreement with the result of the density gradient zone electrophoresis experiment described below (see Fig 3) Based on these results, the toxicity must be supposed to be in the middle of the three fractions

*Density gradient zone electrophoresis* permitted fractionation of the toxin and then measurement of the activity in the various fractions For this purpose an LBK electrophoresis apparatus was used The gradient was made up from saccharose dissolved in Tris buffer with a pH of 8 9 The tension was 1 5 V cm An amount of 3 5 ml of the partly purified toxin Fj A was used After electrophoresis for 26 hours the gradient with the toxin was passed through an ultraviolet absorption meter at 254 mμ and the transmission curve shown in Fig 3 A was registered After having passed the absorption meter the fluid was col-

## OWN EXPERIMENTS

Weeverfish toxin extract is an almost colourless fluid with slightly increased viscosity and pH about neutral point. The toxic part is not dialysable since after dialysis in cellophane tubing for 24 hours at +4° C against physiological saline it is found within the membrane.

The dry weight of one of the extracts, Fj II, was found, after drying to constant weight at 100° C, to be 13.2 mg/ml excluding the NaCL originating from the physiological saline used for extraction.

The total nitrogen in the same preparation was found by the Kjeldahl method to be 2.1 mg per ml and the protein nitrogen 1.58 mg per ml (determined by Kjeldahl analysis of the repeatedly washed sediment after addition of trichloracetic acid). On the basis of a nitrogen content in the protein of 16 per cent, as in the majority of genuine proteins this would correspond to 9.9 mg protein per ml, or about 75 per cent of the dry substance.

Since Fj II contained 1,280 DML per ml, this is equivalent to about 130 DML per mg protein.

*Precipitation* of the toxin with $(NH_4)_2SO_4$ was carried out at a pH of approximately 6. Preliminary experiments showed that addition of 20 W/v per cent solid $(NH_4)_2SO_4$ caused precipitation of a fraction which could not, or at any rate to only a slight extent, be redissolved. This was irrespective of whether precipitation was carried out at room temperature or at 4° C. The fractions precipitated by 20–24 per cent and 24–28.8 per cent redissolved easily and contained almost all of the toxic protein which could be recovered after precipitation. In fraction 24–28.8 per cent there was almost twice as much of the toxic activity as in fraction 20–24 per cent, while in the filtrate from 28.8 per cent precipitation only traces of the toxicity remained.

In subsequent experiments the fraction which precipitated between 20 and 30 per cent AmSo₄ was isolated. It appeared to contain only half the original amount of protein per DML. However the loss by this procedure was so great (more than 70 per cent) even when precipitation was carried out at +4° C that the method had to be abandoned because of lack of material.

*Spectrophotometry* of a crude extract, Fj X containing about 600 DML and 10 mg protein per ml, corresponding to about 60 DML per mg protein gave the absorption curve shown in Fig. 1 B.

When the weever toxin, which had been frozen for some time was thawed and allowed to stand at room temperature a copious sediment appeared. This occurred without loss of toxicity provided the toxin did not stand for longer periods. Spectrophotometry of the supernatant from such a preparation which had been kept for 14 days at room temperature gave the curve shown in Fig. 1 C. This curve has a considerably lower position than curve B and a "purer" protein form. When read at 278 mμ it can be seen that about half the protein in the original solution

fraction 16 and decreases evenly to both sides Protein determination of that fraction showed 0 58 mg protein per ml, corresponding to 275 DML per mg protein Thus the specific toxin seems to account for no more than about 25 per cent of the protein in the original crude material

## IMMUNIZATION EXPERIMENTS

A few workers tried at an early stage to immunize animals with weever toxin *Briot* (1902) carried out the hitherto most comprehensive experiments in that field He was able to protect rabbits against two lethal doses of toxin by means of two subcutaneous injections of a sublethal dose In addition, he proved that it was possible to protect rabbits passively against small doses of toxin by injecting them beforehand with the serum from actively immunized rabbits

In order to investigate further the antigenic properties of weeverfish toxin, the author has carried out a number of experiments on rabbits, guinea pigs, and mice with crude or partly purified weever venom with or without the addition of $Al(OH)_3$ When $Al(OH)_3$-gel (*Hansen* 1941) was used, it was added in amounts corresponding to 1 mg Al per ml in the final mixture, by which means 50 per cent of the toxin was adsorbed to the hydroxide

Detoxication of the toxin with various formalin concentrations was carried out followed by examination of the immunizing ability of the detoxicated preparation on mice, both with and without the addition of $Al(OH)_3$

Blood samples were taken from the rabbits and guinea pigs at different times during the immunization and the antibody in the serum was assayed on the basis of its ability to neutralize a certain amount of toxin

For this purpose a series of mixtures were prepared, containing a constant amount of toxin corresponding to a final concentration of approximately 20 DML per ml and 2-5 stage dilutions of serum After standing for an hour at room tem
injected intravenously
two mice per mixture
serum titre is calculated as the number of lethal doses which 1 ml of undiluted serum would neutralize

On mice, the degree of immunity was either measured indirectly as mentioned above, or

Some examples of

and mice are given i

*Rabbits* Three rabbits weighing 2 kg were injected subcutaneously with 0 5 ml of crude weever toxin with $Al(OH)_3$ Ten days later they were given 2 ml of formalin treated toxin and four weeks later a series of injections of toxin preparation F) A without $Al(OH)_3$ The injec-

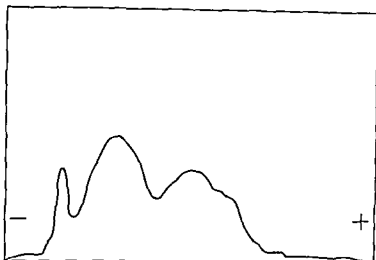



*Fig 2*

Paper electrophoresis of I J A in Tris buffer, pH 8 9, 0 25 mA/cm, 90 V (2 2 per cm) for 18 hours Staining amido black The ordinate is proportional to the extinction and the abscissa to the mobility of the components

lected in 5 ml fractions numbered 9 to 41 in an automatic fraction collector

The curve shows three larger tops corresponding to fractions 9, 16, and 20, all of which gave protein reaction when tested with sulphosalicyclic acid Three lesser tops can be seen at fractions 29, 36, and 41 Toxicity determinations on mice were carried out on fractions 13 to 20 *and 85 per cent of the original amount of toxin was found in fractions* 14 to 19 The activity curve B in Fig 3 has been compiled on the basis of these measurements It will be seen that most of the activity lies in

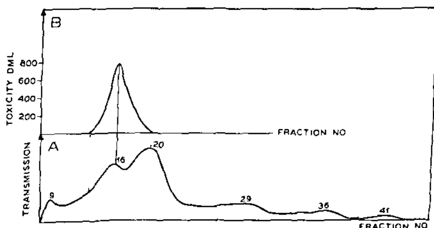



*Fig 3*

A Transmission curve at 254 mμ from density gradient zone electrophoresis of I J A Saccharose Tris buffer, pH 8 9 300 V (1 5 V per cm) 26 hours B Toxicity curve drawn on the same abscissa as in curve A *The abscissa shows the number of the fractions* The ordinate shows the transmission for curve A and the total number of DML per fraction for curve B

TABLE 2

*Immunization of Guinea Pigs with Weever Toxin with and without $Al(OH)_3$*

| Guinea pig no | $Al(OH)_3$ | No of inj | Amnt of protein in mg | Serum* titre 1½ mths | No of inj | Amnt of protein in mg | Serum titre 3 mths | No of inj | Amnt of protein in mg | Serum titre 4 mths | No of inj | Amnt of protein in mg | Serum titre 6 mths | Total no of inj | Total amnt of protein in mg | Serum titre 7½ mths |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4921 | + | 3 | 50 | > 320 < 620 | 3 | 50 | > 40 < 160 | 4 | 70 | > 40 < 160 | 4 | 70 | > 60 < 240 | 5 | 75 | > 240 < 480 |
| 4922 | + | 3 | 50 | > 320 < 620 | 3 | 50 | > 40 < 160 | 4 | 70 | > 160† < 320 | | | | | | |
| 4923 | — | 11 | 120 | < 80 | 11 | 120 | < 40 | 12§ | 140 | < 40 | 12 | 140 | < 60 | 13 | 145 | > 60 < 240 |
| 4924 | — | 11 | 120 | < 80 | 11 | 120 | < 40 | 12§ | 140 | < 40 | 12 | 140 | < 60 | 13 | 145 | > 60 < 240 |

* No of DMI (mice) which can be neutralized by 1 ml guinea pig serum
† Died following heart puncture
§ 10 per cent $Al(OH)_3$ added also to guinea pigs 4923 and 4924

tions in this series were given approximately every third day in doses rising from 0 5 to 1 4 ml

Blood samples were taken 2, 2½ and 3 months after the first injection The titres of these, together with the corresponding total number of injections and total amount of protein injected, are recorded in Table 1

TABLE 1
*Immunization of Rabbits with Weever Toxin*

| Rabbit no | No of inj | Amount of protein in mg | Serum titre* 2 mths | No of inj | Amount of protein in mg | Serum titre 2½ mths | Total no inj | Total amount of protein in mg | Serum titre 3 mths |
|---|---|---|---|---|---|---|---|---|---|
| 4893 | 8 | 72 | > 40 < 200 | 13 | 122 | > 80 < 400 | 16 | 164 | > 320 < 480 |
| 4894 | 8 | 72 | > 200 < 320 | 13 | 122 | > 80 < 400 | 16 | 164 | > 320 < 480 |
| 4895 | 8 | 72 | > 200 < 320 | 13 | 122 | > 80 < 400 | 16 | 164 | > 480 < 640 |

* No of DML (mice) which can be neutralized by 1 ml rabbit serum (see text)

It will be seen that all three rabbits produced neutralizing antibodies and that most of the immunity achieved during the experiment was reached after eight injections with a total of 72 mg protein Only a very moderate increase in titre occurred after an additional eight injections

*Guinea pigs* Four guinea pigs weighing 380 400 grams were injected subcutaneously with weever toxin Fj A Two were given toxin with $Al(OH)_3$ and two others toxin without any $Al(OH)_3$, except for one single injection In the first two, the dose injected was 1 2 ml, at the beginning at about 14 day and later at longer intervals The other two were injected with doses of 0 5 to 2 0 ml, at the beginning at 3 to 4-day and later at longer intervals

Blood specimens were taken 1½, 2, 3 4, 6 and 7½ months after the first injection The results are shown in Table 2

It will be seen that the highest immunity was acquired by the guinea pigs which were given toxin with $Al(OH)_3$ The maximum titres were found after 1½ months as the result of only three injections, after which a decrease occurred during the course of the following six weeks After one and two injections respectively the titres again increased slightly In the two animals which up to the eleventh injection had been given toxin alone, the titres were low during the whole period, but after two injections of toxin with $Al(OH)_3$ an increase occurred in both the animals

*Mice* Different concentrations of formalin were added to untreated weever toxin, after which the pH was adjusted to neutral point with NaOH and the mixtures allowed to stand at room temperature The

TABLE 3

*Immunization of Mice with Detoxicated Weever Toxin with and without Al(OH) and with Corresponding Toxin*

| | No. of inj | Total amnt of protein in mg | Time in days | Degree of immunity | | | |
|---|---|---|---|---|---|---|---|
| | | | | Serum titre | Toxin challenge | | |
| | | | | | No. of mice† | Dose | Observations |
| Detoxicated weever toxin plus $Al(OH)_3$ | 3 | 15 | 60 | < 40* | 2 | 2 DMI § | No reaction |
| Detoxicated weever toxin | 11 | 22 | 60 | < 40 | 2 | 2 DMI | No reaction |
| | | | | | 2 | 4 DMI | Both died |
| | | | | | 2 | 8 DMI | Both died |
| Toxin | 11 | 22 | 60 | > 40 | 2 | 4 DMI | No reaction |
| | | | | | 2 | 8 DMI | No reaction |

* The figures give the number of DMI neutralized by 1 ml of a pool of serum from six mice

† Six mice were omitted from the experiment on account of paravenous injection or death from other causes

§ The figures give the number of DMI injected intravenously into the immunized mice

the mice immunized with the detoxicated preparation, but again to a lesser degree than in the group injected with toxin This indicates that some of the antigenicity was lost during detoxication

Capillary ring tests were carried out on some of the sera of the immunized animals for demonstration of precipitating antibodies Agreement was found between the titres of the precipitating and the neutralizing antibodies

## DISCUSSION AND CONCLUSION

The present study shows that only part of the protein in the crude extract possesses toxic activity and that it is possible by means of various physico-chemical methods to eliminate some of the non poisonous proteins However, the remaining toxic fraction is probably still not the pure toxin

It has been confirmed that weever toxin possesses considerable antigenic properties which can be activated by means of the addition of aluminium hydroxide

It has been shown that the traditional method for detoxicating biological toxins by means of formalin treatment can also be applied to weever toxin, but that its antigenic properties are decreased considerably under the experimental conditions used It may be that given oth[illegible]

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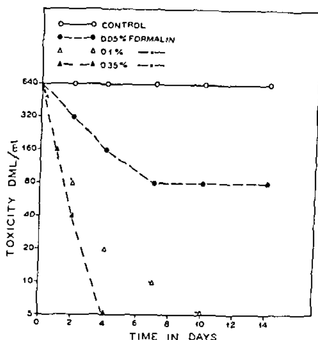



*Fig 4*

Detoxication of weeverfish toxin with various concentrations of formalin The abscissa gives the time in days *from commencement of detoxication The ordinate* gives the number of DML per ml toxin

toxicity was measured intravenously on mice at various times after the addition of formalin The result is shown in Fig 4

It will be seen that detoxication occurs more rapidly the higher the concentration of formalin

The preparation, which had stood for 14 days with 0 1 per cent formalin, was used for immunization of mice in order to ascertain whether the antigenicity was maintained after detoxication It contained < 2 5 DML per ml mice tolerating 0 4 ml intravenously without any reactions Three groups of 12 mice were given subcutaneous injections of either the detoxicated preparation with and without $Al(OH)_3$ or of the corresponding toxin without $Al(OH)_3$ The first group received three weekly injections of 0 5 ml, corresponding to a total of 15 mg protein, and the two other groups a total of 22 mg protein administered in doses of 0 2 ml every third day After 60 days heart puncture was made in half the mice in each group and the antibodies in pools of their serum were measured as described previously The remaining mice were injected intravenously with varying amounts of toxin and observed for 24 hours The results are shown in Table 3

The titres of the pooled blood from the mice treated with detoxicated weever toxin were less than 40, while the titres of the serum from the group treated with toxin were more than 40 DML per ml In both cases the small amount of serum prevented further determination

However, the toxin challenge demonstrated some immunity also in

# BRIEF REPORT

## THE PLACENTAL TRANSFER OF INSULIN ANTIBODIES

By *Jan I Thorell*

### *Methods*

Adult female guinea pigs were given daily injections of insulin NOVO Ultralente subcutaneously the first week 5 IU and later on 10 IU a day Six weeks later they were mated with untreated animals The injections were continued during the first 6 weeks of pregnancy

The fetuses were extracted via an abdominal incision 6 weeks before term and a few days before term On the first occasion blood was obtained through decapitation and pooled from the whole litter On the later occasion blood was obtained from the umbilical cord and from cardiac puncture Blood was obtained from the mothers at the same time

sulin I131 to different serum fractions was localized by electrophoresis The counting rates on the strips were recorded in a continous scanning apparatus with double GM tubes

### *Results*

By electrophoresis the bound insulin I131 migrated with the faster parts of the γ globulin

### *Discussion*

A characteristic change in the infant of the diabetic mother is the

but would crave large amounts of weever toxin and would involve great practical difficulties

## SUMMARY

A number of experiments have been carried out which show that weeverfish toxin is a protein

*Purification of the crude toxin has been possible to some extent by* various methods

Immunization experiments have been carried out on animals in order to examine the antigenicity of weever toxin after treatment of various kinds

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the same time

The insulin antibody titre was assayed with $I^{131}$ tagged insulin by the hydrodynamic flow separation technic (4) 0 1 ml of serum or serum diluted with barbital buffer pH 8 5 was incubated with 0 01 mg (0 01 ml) insulin $I^{131}$ during 30 minutes at room temperature Antibody bound insulin was then separated from unbound insulin through hydrodynamic flow paper chromatography on Whatman 3 MM paper in barbital buffer pH 8 5 during 6 hours where bound insulin migrates with the proteins and unbound insulin remains at the application point The binding of insulin $I^{131}$ to different serum fractions was localized by electrophoresis The counting rates on the strips were recorded in a continous scanning apparatus with double GM tubes

### *Results*

insulin

By electrophoresis the bound insulin $I^{131}$ migrated with the faster parts of the γ globulin

### *Discussion*

Received 30 viii 62 from the Institute of Pathology University of Uppsala Sweden (Head G Hultquist)

Supported by grants from the *Swedish Medical Research Council* and the *Swedish Diabetes Federation*

shows the same characteristics as the insulin binding reaction in insulin treated human subjects Human and guinea pig insulin antibodies crossreact with insulin from several species Thus human beef pork insulin antibodies bind human insulin I131 *in vitro* (5) and guinea pig beef insulin antibodies neutralize endogenous rat insulin *in vivo* (1) Human exogenous insulin antibodies probably bind human endogenous insulin too This is however thought to be of no importance in the uncomplicated diabetes case The observed passage of insulin antibodies from mother to fetus might be the passage of one of the above mentioned antagonists which could influence upon the fetal insulin especially as the fetal antibody titre exceeded that of the mother

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FROM THE CONNECTIVE TISSUE RESEARCH LABORATORY, RIGSHOSPITAL DEPT II AND THE ZOOPHYSIOLOGICAL LABORATORY A UNIVERSITY OF COPENHAGEN

# MITOTIC DIVISION OF TISSUE MAST CELLS AS INDICATED BY THE UPTAKE OF TRITIATED THYMIDINE[1]

By

G Asboe-Hansen and Hilde Levi

Received 13 iv 62

The regeneration of tissue mast cells is a standing matter of dispute Although mast cell infiltration of dermis, lymph glands, bone and bone marrow, spleen, liver, lung etc is well known, most investigators agree that mitotic figures are rarely seen in mast cells of the adult organism (*Downey* 1913, *Padawer* 1960) Various findings indicate that amitotic division occurs in these cells (*Michels* 1938), and several authors believe in heteroplastic regeneration of mast cells from lymphocytes (*Sabrazes & Lafon* 1908), plasma cells (*Michels* 1935), histiocytes (*Herzog* 1916), and fibroblasts (*Bates* 1935) *Holmgren* (1946) assumed that mast cells develop from perivascular mesenchymal cells and that they follow their own line of development, a notion that has been widely accepted

The fact that the nucleus is mostly concealed by the cytoplasmic granules has undoubtedly interfered with some workers' efforts to demonstrate mitotic figures in mast cells *Hunt & Hunt* (1957), after degranulation of mast cells by compound 48 80, found an entirely reasonable number of mitoses in mast cells

Mitosis is known to be preceded by DNA synthesis After incorporation of a labelled DNA precursor, it becomes possible to recognize the labelled nucleus in autoradiograms The nucleus may be labelled even before the morphologic phenomenon of mitotic division takes place However, the nucleus keeps its label during mitosis and passes [illegible] the next [illegible]

ce [illegible]

a [illegible]

p [illegible] the mentioned requirements DNA is almost selectively labelled and the low beta ray energy of tritium ensures

[1] These investigations were supported in part by the *Danish Anti Cancer League*, *Richardson Merrell Inc* USA, and the *James Picker Foundation* (*National Academy of Sciences National Research Council*)

shows the same characteristics as the insulin binding reaction in insulin treated human subjects Human and guinea pig insulin antibodies crossreact with insulin from several species Thus human beef pork insulin antibodies bind human insulin $I^{131}$ *in vitro* (5) and guinea pig beef insulin antibodies neutralize endogenous rat insulin *in vivo* (1) Human exogenous insulin antibodies probably bind human endogenous insulin too This is however thought to be of no importance in the uncomplicated diabetes case The observed passage of insulin antibodies from mother to fetus might be the passage of one of the above mentioned antagonists which could influence upon the fetal insulin especially as the fetal antibody titre exceeded that of the mother

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high resolution of the autoradiograms Tritiated thymidine has been shown to be available in the body for about 30-60 minutes after injection (*Cronkite et al* 1959) During this period it is either degraded or incorporated into DNA

## EXPERIMENTAL

White female mice of the strain St/EH[2], six weeks old, were painted four times with 0 5 ml of a 0 5 per cent benzene solution of the carcinogenic hydrocarbon 9 10 dimethyl-1,2 benzanthracene After six to seven weeks, 10 per cent of the painted mice had developed one or more papillomas on their back skin In histological sections of the induced skin tumours dense accumulation of mast cells were demonstrated

The tumour bearing mice were injected intraperitoneally with 5 microcuries of tritiated thymidine per gram body weight The injections were given 8 10 days after at least one papilloma had been observed on the back skin

One half to twenty hours after the injection, the animals were sacrified the tumours together with surrounding skin were excised and fixed in a solution consisting of absolute alcohol 85 per cent , formaldehyde 10 per cent and glacial acetic acid 5 per cent Thereafter, the tissue was embedded in paraffin and sections were cut at 7 micra's thickness After deparaffinization autoradiograms were made using Kodak AR 10 stripping film After 14 days' exposure in the cold the slides were processed and subsequently stained with a 0 05 per cent aqueous solution of toluidine blue for 60 seconds

Mast cells could be identified easily in the mounted slides due to the purple metachromatic staining of the numerous cytoplasmic granules The cell nucleus appeared more or less clear of granules

Since the average range of beta particles emitted by tritium is only one or two micra in a medium of density 1, a tissue section of 7 micra s thickness represents an infinitely thick sample Only the uppermost layer of the section will contribute to the autoradiographic picture When estimating the fraction of labelled cells in the tissue section the number of inactive cells will thus be grossly overestimated because the cells situated in the central or deeper part of the section cannot produce an autoradiogram The use of thinner sections was found to be disadvantageous because mast cells were torn badly granules and cytoplasm were smeared out and therefore a correlation between emulsion blackening and histological detail became ambiguous An evaluation of grain counts over individual cells with the use of 7 μ sections and AR 10 stripping film leads to a minimum figure for the number of labelled cells but it provides a possibility for comparing rather than determining labelling indices at different time intervals between injection of the precursor and sacrifice

## RESULTS

The autoradiograms showed marked labelling of connective tissue and epithelial cells as early as 30 minutes after injection of tritiated thymidine (Fig 1) Blackening was located primarily over cells, and very few grains were observed over extracellular substance In all preparations studied, radioactive mast cells were found Some of these gave rise to blackening of the emulsion over the cell nucleus while the cytoplasm appeared free from silver grains In other cells, however, both the nucleus and the cytoplasm seemed to be labelled, and even the appearance of silver grains over cytoplasm, surrounding an apparently unlabelled nucleus, was not infrequent This observation may well be

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[2] Our thanks are due to *Jorgen Kieler* M D the Fibiger Laboratory for Cancer Research for supplying the experimental animals



*Fig 1*

Aut rad gram Several ep dermal cells (orthochromatic H e) and three mast cells (metach omat c purple) showing nuclear lat ell ng w th H3 thym dine Stain 0 05 per cent aqueous solut ons of t luidine blue for one m nute Magnification 1300 ×

due to the fact that the strong metachromatic staining of the cyto plasmic granules makes accurate distinction of boundaries and the localization of different nuclear constituents difficult Moreover the heavy granularity of the cells interfered with the counting of grains in the emulsion Where the interpretation was doubtful the lowest grain count was accepted so that the tendency is toward an underestimate of the number of silver grains observed

On each autoradiogram at least 1000 cells were carefully examined Between 2 and 3 per cent of these cells were labelled significantly above background Roughly 1 per cent showed significant labelling of the nucleus and 1 2 per cent showed significant labelling of the cytoplasm Preparations representing 6 different intervals within twenty hours between injection and sacrifice showed very similar grain counts so that a change in the number of labelled cells as a function of time could not be established with certainty

## DISCUSSION

In view of the imperfections of the techniques used exact figures for the percentage of labelled cells and for the cellular distribution of the labelled compound (DNA) can not be given However it seems safe to conclude from the above observations that at least 1 per cent of the

mast cells of the connective tissue below induced epidermic papillomas have incorporated *tritiated thymidine in the course of 30–60 minutes*, during which period the precursor is available In the majority of the studies described in the literature, DNA synthesis occurs in cell nuclei and labelling with a DNA *precursor manifests itself in labelled nuclei* However, some exceptions from this rule are known

Experiments are in progress to clarify these problems and to determine the mitotic cycle of mast cells in tissue sections and in cell suspensions

## SUMMARY

Mast cells of the connective tissue below carcinogen-induced epidermic papillomas of white female mice were studied by autoradiography after intraperitoneal *injection of tritiated thymidine* 1–3 *per* cent of the cells were labelled above background within the period of 0 5 and 20 hours, indicating DNA synthesis and mitotic division of mature granulated mast cells

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# THE INFLUENCE OF ESTROGENIC AND ANDROGENIC HORMONES ON MAST CELLS AND CONNECTIVE TISSUE IN UTERUS OF GUINEA PIG

*By*

OLE H. IVERSEN

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A previous publication (5) presented the results of the effect of estrogenic and androgenic hormones on the mast cells in the human cervix uteri. The object of the present study has been further to investigate any changes which may occur in the morphology and quantity of mast cells in the uteri of spayed guinea pigs during estrogenic and androgenic treatment and to study any histochemical variations which may take place in the connective tissue under such conditions.

## MATERIAL AND METHODS

The study comprises 34 female guinea pigs weighing from *600 to 700 g*. Out of these 5 were used for controls and killed by ether without any previous therapy. 29 were oophorectomized under narcodorm ether anaesthesia. Four to six weeks later samples of vaginal secretion were drawn for control. Thereupon the animals were divided into groups:

1) 6 animals were killed by ether without any treatment.

2) 10 animals were treated with estradiol dipropionate (Diprovex Leo ®)—0.25 mg subcutaneously every second day for a total of four times. These animals were killed 2 days after the last injection.

3) 7 animals were treated with testosterone propionate—2.5mg subcutaneously every second day, a total of 4 injections, and killed 2 days after the last injection.

4) 6 anim... [illegible]

... cut in sections at 5 micra.

The following staining have been employed:

*Formalin fixed specimens*: Periodic acid Schiff's staining (PAS). Alcain blue staining pH 2.5 (13). Hale's staining (4). Hale's staining combined with PAS (Mowry) (10). van Gieson Hansen's staining. Haematoxylin eosin staining. Unna Pappenheim.

*Lead acetate fixed specimens*: Toluidine blue staining pH 4.5.

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Aided by grants from the *Danish League against Rheumatism* and the *Danish State Science Foundation*.

*Calcium acetate-formalin fixed specimens* *Spicer's* Alcian blue safranin staining (12)

Several of the specimens from the various groups were incubated with bacterial hyaluronidase (supplied by *Dr V Faber* of the Danish State Seruminstitute Copenhagen) and with testicular hyaluronidase (Invasin Lundbeck ®) The enzyme was dissolved in McIlvaine's Standard Buffer (pH 6 5) and the incubation was performed at 20° C for one hour Furthermore incubation with ribonuclease and diastase accompanied by control with McIlvaine's Standard Buffer was performed After incubation, the specimens were stained with toluidine blue, PAS and Hale s stain

## RESULTS

Macroscopically, the uteri of the spayed animals appeared to be distinctly smaller than in normal animals, the horns were more gracile and pale and of a firmer consistency Following estrogenic therapy, a pronounced increase in the thickness and length of the horns was found, the colour was more reddish cyanotic and the tissue softer and more oedematous After androgenic therapy, some increase in thickness and length did also appear, but the colour became more pink and the tissue firmer than in the animals treated with estrogens

### *Mast Cells*

As in other species of animals, the mast cells in the uterus of the guinea pig are scarce in the basal parts of the endometrium, while the myometrium contains very considerable quantities of mast cells, especially in the septa of connective tissue and more particularly congested around the vessels In the myometrium no difference between the cervix and the horns was observed In intact animals, the mast cells were relatively large and well granulated, the granules showed beautiful metachromatic staining (Figs 1 and 2) In the spayed and untreated

*Figs 1–8*

*Fig 1* Cervix uteri from normal untreated guinea pig (Toluidine blue × 100) Mast cells in the endometrium and the myometrium

*Fig 2* Cervix uteri from normal untreated guinea pig (Toluidine blue × 400) Mast cells with dense orthochromatic granulation

*Fig 3* Cervix uteri from an oophorectomized guinea pig following estrogenic therapy (Toluidine blue × 128) Scattered mast cells with metachromatic clouds The density of the stroma cells is less pronounced than in untreated animals

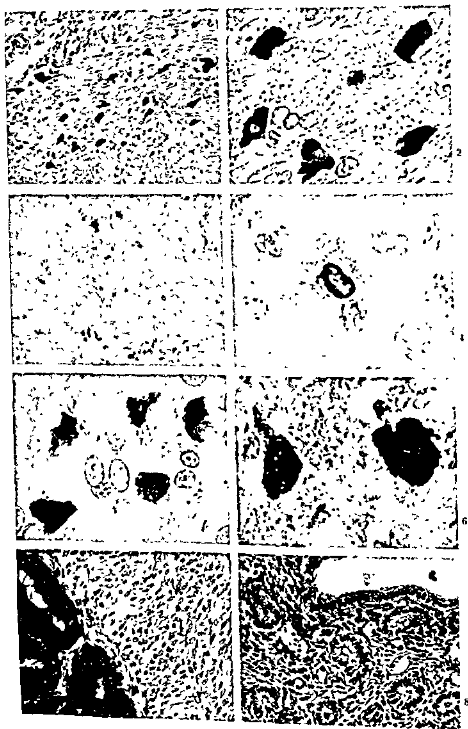
*Fig 4* High power view (Toluidine blue × 520) Mast cells from cervix uteri (an *oophorectomized guinea pig treated with estrogens*) showing degranulation and metachromasia of the granules

*Fig 5* Cervix uteri from an untreated oophorectomized guinea pig (Toluidine blue × 100) Mast cells with relatively dense and dark metachromatic granulation

*Fig 6* High power view (Toluidine-blue × 400) of mast cells from cervix uteri (an oophorectomized guinea pig treated with androgens) showing large mast cells with dense orthochromatic granulation

*Fig 7* Cervix uteri from an oophorectomized guinea pig after estrogenic therapy (PAS × 100) Strong PAS reaction in the upper layers of the endometrium weaker reaction in the lower layers of the endometrium and in the interstitial connective tissue of the myometrium

*Fig 8* Cornu uteri from an oophorectomized guinea pig after androgenic therapy (Hale × 100) Positive Hale reaction in the stroma of the endometrium




Figs 1–8

animals, the mast cells appeared to be of nearly the same size, but the *granules were denser and more blue-violet of colour (Fig 5) No difference* in the number of mast cells in the two groups was observed

After treatment with *relatively low doses of estrogens, a considerable* decrease in the number of mast cells was found, both in the endometrium (where *now only scanty amounts of mast cells were present)* and in the connective tissue of the myometrium The cells were distinctly degranulated, metachromatic clouds or halo became extremely pronounced and vacuoles were formed in the cytoplasm (Figs 3 and 4) The metachromasia was light red, lighter than in normal animals In the animals treated with androgens, both quantitative and morphological changes reappeared The number of mast cells increased in spite *of decreasing density of cells in the tissue The mast cells were large,* the granules were well defined and dark, almost orthochromatic No degranulation occurred, on the whole the cells *resembled to a certain extent* the cells in untreated spayed animals, only they were distinctly larger (Fig 6)

In the group comprising intensively estrogen treated animals, a marked increase in the number of mast cells was *observed* Morphologically, however, the cells were larger, the granules were strongly metachromatically bluish red Only a few of the cells were degranulated

In none of the groups, PAS positive mast cells were seen although *staining for up to 12 hours was attempted (Compton (2))* Furthermore, no Hale positive granules were observed in any of the groups After incubation the mast cells disappeared in all of the groups, both after bacterial and testicular hyaluronidase On the other hand only few mast cells were seen after incubation with buffer solution alone

Staining after *Spicer's* method (12) was tried, but no positive reaction was seen in any of the groups

*The Ground Substance*

The most pronounced changes in the connective tissue of the uterine wall (cervix and horns) were those observed by haematoxylin-eosin and van Gieson-Hansen's staining methods, *viz* decreasing density of cells following both estrogenic and androgenic treatment In untreated and spayed animals the muscle fibrils were very compact, but after hormone therapy and particularly after prolonged estrogenic therapy more abundant *collagen was observed in addition to the increasing* oedema (or ground substance) *As regards the remaining methods of* staining, no distinct qualitative differences were observed, whereas quantitatively there was markedly increasing ground substance both after estrogenic and androgenic therapy The conditions were relatively uniform in cervix and horns The metachromasia was equaley pink in controls, in spayed animals, and in hormone treated animals

The Hale reaction appeared to be more positive after hormone treatment, especially after androgenic treatment(Fig 8), and was particularly strong in the horns The PAS reaction was only very slightly positive in all the groups, the colour was pink, resembling the reaction seen in neutral mucopolysaccharides The muscles showed a stronger positive PAS reaction after estrogenic treatment (glycogen?) (Fig 7)

The Alcian stain gave a faintly positive reaction in the controls, and a somewhat stronger reaction in the hormone treated groups

The PAS Hale combination has as usual given very beautiful pictures and has shown the quantitative changes in the ground substance mentioned above

The Unna-Pappenheim stain has shown a number of pyroninophilic cells in relation to increased vascularization during hormone therapy

As regards the nature of the ground substance, the incubation experiments have afforded no unambiguous solution, no pronounced al-
[illegible] the PAS reactions
onidase, testicular

*The Endometrium*

In the controls the endometrium showed slight metachromasia, positive Hale reaction and pink colour after PAS staining In oophorectomized animals the reactions were even weaker After hormone therapy the metachromasia and the Hale reaction were stronger, while the PAS reaction appeared to be unchanged The metachromasia and the Hale reaction did not change after incubation with ribonuclease, bacterial hyaluronidase and testicular hyaluronidase

DISCUSSION

The present investigations show that pronounced morphological and quantitative changes of the mast cells occur in the uterus of guinea pig, both in the cervix and in the horns As to the quantitative alterations during brief estrogenic treatment the present results are consistent with investigations carried out by *Johansson & Westin* (7) In the horns of spayed mice, they found after a single injection of estrogens a significant decrease in the number of mast cells 24 hours after injection slowly increasing to a little below normal values in the course of 10 days A marked increase in the number of mast cells [illegible]

[illegible] of the author's previous investigations (*Iversen* 1960 (5)) A significant decrease in the number of mast cells in the human cervix uteri following a short period of estrogenic therapy

*The morphological changes, the degranulation, appear clearly from the experiments and the illustrations Comparison with the mast cells in the remaining experimental groups rules out definitely the possibility of artificial changes, as suggested inter alia by Dewitt et al*, and *Compton* (3, 2) *It is significant that the degranulation appears primarily in tissue treated with small doses of estrogens, while the mast cells from animals treated with large doses much more resemble the cells of the androgen group In this case the metachromasia is not so pronounced, the colour becoming more orthochromatic It may well be that the cells of these groups should be regarded as more "immatur" (Riley type I?), but I have not as *Riley* (11) found increased amounts of PAS positive mast cells

Contrary to previous statements in the literature, *Kellsall & Crabb* (8) *and Lindner* (9), *I have found distinct morphological changes after androgenic therapy, almost corresponding to the changes which I previously observed in a human material In the human cervix I found a decrease in the number of mast cells after androgenic therapy, in the guinea pig uterus an increase The difference may be due to differences in the dosage of hormones, differences in the tissue, or simply the phenomenon which is so often seen, that mast cells behave differently in various species of animals, and even in various kinds of tissue

The incubation experiments performed exclude the ribonucleic acid as a component of the granules and the ground substance *Incubation with* hyaluronidase shows no greater variations as regards the ground substance *The latter phenomenon may be due to the fact that the muco* polysaccharides present primarily are sulphated mucopolysaccharides, but it may also indicate that the mucopolysaccharides occur in low concentrations only The present experiments do not allow of any conclusions as to the degree of polymerization In a previous study on human cervix uteri the author has shown (6) that during estrogenic therapy a change in the ratio between hyaluronic acid and chondroitin sulphate takes place while no *polymerization of the hyaluronic acid* occurs

*The changes in the endometrium corresponds to those presented by Zachariae* (14) In the corpus endometrium of the human uteri, he found a pronounced metachromasia localized to the luminal two thirds of the endometrium following estrogenic therapy, the metachromasia being insensitive to testicular hyaluronidase

*It should be observed that pyroninophilic cells were found* in the groups treated with hormones indicative of a protein synthesis, and that some *of the groups treated with hormones present the* Kurloff bodies (*Christensen, Hjort & Iversen* 1962 (1)) Furthermore, it is worth noting that we did not succeed in colouring the mast cells in specimens of guinea pig tissue by means of Spicer's Alcian blue-safranin stain We cannot offer any explanation of the latter phenomenon

Without expatiating on the many different theories as to mast cell

function it must be concluded on the basis of the present investigation that distinct changes in the conditions of the mast cells occur both quantitatively and morphologically under the non physiological conditions created by the large doses of hormones employed in the present study Furthermore it can be concluded that during estrogenic therapy a considerable mobilization occurs of connective tissue ground substance and fibrils in the uterus of guinea pig


## SUMMARY

The influence exerted by estrogens and androgens on the morphology and number of mast cells and on the histochemistry of the connective tissue in the uteri of guinea pigs is investigated Brief estrogenic therapy reduces the number of mast cells and a considerable degranulation occurs Prolonged estrogenic therapy increases the number the mast cells do not degranulate and they are larger than in the control animals Androgens increase the number of mast cells the cells are larger than in the controls and almost orthochromatic Histochemical studies indicate an increase in acid mucopolysaccharides in the endometrial stroma both under estrogenic and androgenic therapy Furthermore the investigations show that the interstitial connective tissue of the myometrium increases under estrogenic and androgenic therapy The histochemical studies do not permit of any definite conclusions as to the character of the ground substance but suggest that a mobilization possibly a synthesis of acid mucopolysaccharides occurs The histochemical reactions are insensitive to ribonuclease bacterial hyaluronidase and testicular hyaluronidase


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The morphological changes, the degranulation, *appear clearly from* the experiments and the illustrations Comparison with the mast cells in the remaining experimental *groups rules out definitely the possibility* of artificial changes, as suggested inter alia by *Dewitt et al* and *Compton* (3, 2) *It is significant that* the degranulation appears primarily in tissue treated with small doses of estrogens, while the mast cells *from* animals treated with large doses much more resemble the cells of the androgen group In this case the metachromasia is not so pronounced, the colour becoming more orthochromatic It may well be that the cells of these groups should be regarded as more "immatur' (Riley type I?), but I have not as *Riley* (11) found increased amounts of PAS positive mast cells

Contrary to previous statements in the literature, *Kellsall & Crabb* (8) and *Lindner* (9), I have found distinct morphological changes after androgenic therapy, almost corresponding to the changes which I previously observed in a human material In the human cervix I found a decrease in the number of mast cells after androgenic therapy, in the guinea pig uterus an increase The difference may be due to differences in the dosage of hormones, differences in the tissue, or simply the phenomenon which is so often seen, that mast cells behave differently in various species of animals, and even in various kinds of tissue

*The incubation experiments performed exclude the ribonucleic acid* as a component of the granules and the ground substance Incubation with *hyaluronidase shows no greater variations as regards the ground* substance The latter phenomenon may be due to the fact that the muco-*polysaccharides present primarily are sulphated mucopolysaccharides*, but it may also indicate that the mucopolysaccharides occur in low concentrations only *The present experiments do not allow of any* conclusions as to the degree of polymerization In a previous study on human cervix uteri *the author has shown (6) that during estrogenic the*rapy a change in the ratio between hyaluronic acid and chondroitin sulphate takes place while *no polymerization of the hyaluronic acid* occurs

The changes in the endometrium corresponds *to those presented by Zachariae* (14) In the corpus endometrium of the human uteri, he found a pronounced *metachromasia localized to the luminal two-thirds* of the endometrium following estrogenic therapy, the metachromasia being insensitive to *testicular hyaluronidase*

It should be observed that pyroninophilic cells were found in the groups treated with hormones, indicative of a protein synthesis, and that some of the groups treated with hormones present the Kurloff bodies (*Christensen, Hjort & Iversen 1962 (1)*) Furthermore, it is worth noting that we did not succeed in colouring the mast cells in specimens of guinea pig tissue *by means of Spicer's Alcian blue-safranin* stain We cannot offer any explanation of the latter phenomenon

Without expatiating on the *many different theories as to mast cell*

function it must be concluded on the basis of the present investigation that distinct changes in the conditions of the mast cells occur, both quantitatively and morphologically under the non physiological conditions created by the large doses of hormones employed in the present study Furthermore it can be concluded that during estrogenic therapy a considerable mobilization occurs of connective tissue ground substance and fibrils in the uterus of guinea pig

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# A CASE OF BRENNER TUMOUR IN MALIGNANT TRANSFORMATION

By

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Received 4 x 62

Brenner tumours of the ovary have generally been looked upon as definitely benign neoplasms Within the last two decades, however, a few reports on single malignant cases have appeared (*Dubrauszky & v Massenbach* 1947, *Dubrauszky* 1949, *Rawson & Helman* 1955, *Mac Kinley* 1956, *Bovard et al* 1957) The patients were in the middle or the end of the sixth decade, and clinical symptoms indicated malignant disease Grossly the tumours were unilateral and partly cystic, partly solid The histologic picture showed transition from typical Brenner tissue with cystadenomatous areas into malignant solid or adenocystic tumour tissue In one case (*Rawson & Helman* 1955) the patient died 5½ months after operation with extensive invasion of the pelvic organs and metastases to the left kidney The cystadenomatous part of this tumour had shown to be of the serous variety Also, *Bovard et al* (1957) observed local recurrence with large pelvic tumour masses and peritoneal carcinomatosis

*Novak* (1958) reports two additional cases, one encountered by *Limburg* and illustrated convincingly, the other described by *Sirsat* (1956)

The two tumours of *v Numers* (1945) may be malignant Brenner tumours, but proof is lacking, as no typical benign epithelial nests are left Similarly, the diagnosis in the cases of *Reel* (1958), *Foda & Shafeek* (1959) and *Thoyer Rozat et al* (1960) is questionable

The present report intends to introduce a new case of malignant Brenner tumour

## CASE HISTORY

The patient was an 81 years old woman [illegible]

* The author is indebted to *Professor G Teilum* for help in evaluating the microscopic slides



Fig 1



Fig 2



Fig 3



Fig 4

On admission a large cystic and movable tumour filled the right side of the abdomen No tenderness Nothing was felt in the left side, and gynaecological examination was without abnormal findings

At operation, an unilocular cyst, about 20 cm in diameter, was found arising from the right ovary The left ovary was the seat of a similar cystic tumour, measuring about 7–8 cm Ascites or carcinosis were not noted Both tumours were exstirpated without complications, and the patient could be dismissed 10 days after operation

Grossly, the wall of the right sided cyst measured from one to three millimeters and was externally smooth The inner surface was sprinkled with groups of small warty excrescences On the left side, the cyst was smooth on both surfaces, and the wall had a thickness of about two millimeters This contained a single, firm and elastic, almond-shaped nodule, measuring about 8 × 15 mm and with a whitish, slightly yellow cut surface Tiny cysts might faintly be seen

Microscopically, the larger cyst showed the picture of a papillary serous cystadenoma The lining epithelium, however, was proliferating, thick and stratified with closely packed, large, dark and oval, but slightly irregular nuclei and an occasional mitosis (Fig 1) In one minute area small epithelial clefts were numerous and intermingled with psammoma bodies (Fig 2) Pronounced epithelial atypia or invasive growth could not be demonstrated, and so the tumour seems to belong to the borderline group, intermediate between the frankly benign cystadenomas and the papillary cystadenocarcinomas

The solid part of the left-sided cystic tumour was characteristically *fibroepithelial, made up of a thick and coarse*, richly cellular, fibromatous stroma with rather evenly distributed epithelial islands of varying size and round, oval or more irregular (Fig 3) Some of these nests were typically benign with clear, polygonal or slightly elongated cells and oval nuclei of a striking uniformity and often with a longitudinal grooving (Fig 4) In the central parts of the epithelium small groups of vacuolated cells with an ample, quite clear cytoplasm were common, and these cells lined many small cystic cavities as a cubical or cylindrical pseudomucinous epithelium (Fig 5) The central cystic degeneration in the epithelial islands was pronounced and the small lumina contained eosinophilic precipitates The lining cells were of the above mentioned type, or just flattened epithelial cells Staining for mucin was faintly positive for the precipitates and for a few of the clear cells

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*Fig 1* Papillary serous cystadenoma of the right ovary The lining epithelium shows proliferative activity (100 ×)

*Fig 2* Small area of the papillary cystadenoma with epithelial clefts and psammoma bodies (40 ×)

*Fig 3* Brenner tumour of the left ovary with benign and atypical epithelial islands (10 ×)

*Fig 4* Typical epithelial nest of Brenner tumour (100 ×)



Fig 5



Fig 6



Fig 7



Fig 8

In most of the epithelial nests, however, a gradual or more abrupt transition into a dark, atypical epithelium was seen (Figs 6 and 7) The atypical cells had a scanty, very dark cytoplasm and the nuclei were closely packed, enlarged and hyperchromatic Polymorphism was moderate and mitoses rare In one place, a small solid bud of large twisted cells with ample strongly eosinophilic cytoplasm and pycnotic nuclei pushed into the stroma (Fig 8) Cystic degeneration was as common as in the benign epithelium, but the outline of the cavities was often more irregular Yet the ability to differentiate into clear, cubical cells of the pseudomucinous variety was retained (Fig 9) The cavities, some of them large, contained desquamated dark or vacuolated epithelial cells and eosinophilic precipitates (Fig 10)

The described changes seemed to be most advanced in the peripheral part of the tumor where the whole pattern was more disorderly, and here a few epithelial nests were totally transformed Signs of invasive growth were sparse, but definite In places, the border between epithelium and connective tissue was blurred and here an occasional epithelial string invaded the stroma which in addition contained small, ill-defined and atypical epithelial islands (Fig 11) Most impressive, however, was the finding of tumour emboli in the vessels of the tumour and especially of the looser connective tissue adjacent to it (Fig 12) In these epithelial islands, like in the main tumour, both cystic degeneration and vacuolated cells of the pseudomucinous variety were seen (Fig 13)

The cystic part of the left sided tumour had a fibrous wall and was lined by a flat, dark, uncharacteristic epithelium Near to the solid part, small irregular strings and islands of dark undifferentiated epithelium were invading the connective tissue (Fig 14) The cyst may be supposed to have arisen by a further development of one of the tiny cysts described above

## DISCUSSION

In the present case the diagnosis of malignancy is based upon epithelial atypia with enlargement and hyperchromasia of nuclei abnormal nucleoc[illegible]

The fi[illegible]

lium i[illegible] in the latter of some of the characteristic structural features plainly show the original nature of the tumour Furthermore, the tumour emboli present a complete image of the atypical epithelial nests in the primary tumour and so,

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Fig 5 Cystic degeneration of an epithelial island of the Brenner tumo[illegible] Th[illegible] cavities are lined by a [illegible]

Fig 6 and 7 Epithelial nest[illegible] into dark atypical ep[illegible]

Fig 8 Solid bud of eosinoph[illegible]

Fig 9

Fig 10

Fig 11

Fig 12



*Fig 13* Tumour embolus with tiny cysts, some lined by a clear epithelium some containing eosinophilic precipitates (100 ×)

*Fig 14* Tumour invasion into the connective tissue of the cystic part of the Brenner tumour (160 ×)

secondary extension from the papillary cystadenoma of the other side can be excluded The diagnosis of malignant Brenner tumour, therefore, seems justified

The operation was performed in order to remove the large cyst of the right ovary, and the cystic tumour on the left side was diagnosed for the first time on that occasion This may explain the rather early stage in malignant transformation, and so the opportunity to establish the origin as a Brenner tumour The cases of *v Numers* (1945) are more advanced with no benign elements, and according to *Dubrauszky & v Massenbach* (1947) it cannot be excluded that some solid or cystic carcinomas of the ovary may originate from Brenner tumours Some reports (*Abell* 1957, *Guvener* 1961) may support this view, but *v Numers* (1945) undoubtedly is right in regarding the malignant form of

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*Fig 9* Atypical epithelial nest of Brenner tumour with cystic degeneration The

*Fig*

*Fig*

*Fig*



Fig 9



Fig 10

Fig 11

Fig 12

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# EFFECT OF NICOTINIC ACID AND MAGNESIUM NICOTINATE ON CHOLESTEROL-INDUCED ATHEROSCLEROSIS IN COCKERELS

*By*

JOSEF BURSTEIN and ANTTI TELKKÄ

Received 7 v 62

It is universally recognized that nicotinic acid depresses the elevated serum cholesterol level in man (*Parsons & Flinn* 1957, *Öst* 1960, and *Berge et al* 1961) It is not known, however, whether this decrease may be of value in the prevention or treatment of atherosclerosis Evidence concerning this question from the animal experiments has been limited up to the present *Altschul* (1956) and *Cawa et al* (1959) noticed preventative effect of nicotinic acid on cholesterol-induced atherosclerotic changes in the aorta of rabbit The latter authors stated that the degree of the involvement of the aorta was lower in cholesterol-fed animals treated with nicotinic acid than in those receiving cholesterol alone This difference was even more striking microscopically when the thickness of the plaques in the animals of the two groups was compared

According to *Cawa et al* (1959) pathologic changes due to the possible toxicity of nicotinic acid were not found except for a few haemorrhagic lesions in the kidney Fatty deposition in the liver, of animals fed with nicotinic acid was slightly more pronounced than that of animals fed with cholesterol only

*Merril & Lemley-Stone* (1957) analyzed the cholesterol content of the aorta and of the liver in cholesterol-fed rabbits and that of the liver in cholesterol fed rabbits treated with nicotinic acid, they noticed that nicotinic acid effectively inhibited the deposition of cholesterol in the aorta and to a lesser degree, the cholesterol storage in the liver

*Gaylor & coworkers* (1960) succeeded in suppressing the degree of elevation of blood cholesterol in cholesterol fed chicks treated with nicotinic acid whereas nicotinic acid produced a substantial rise of liver cholesterol Fatty infiltration of the liver, as a result of nicotine amide treatment, has been reported by *Handler & Dubin* (1946) According to a recent report by *Hunter & Wong* (1961) nicotinic acid completely failed to prevent the cholesterol-induced atheromatous changes

Brenner tumour a rarity, considering the relatively low incidence of the benign form and the infrequent malignancy of the comparative pseudomucinous cystadenoma

*Dubrauszky* (1949) describes areas of incipient squamous metaplasia in the malignant Brenner tumour, and the case of *MacKinley* (1956) was in part "almost epidermoid in type" These findings may correspond to the small bud of eosinophilic cells in the present tumour

## SUMMARY

A case of malignant Brenner tumour in an 81 years old woman is described Symptoms were largely due to a large serous cystadenoma of the other ovary Gross examination of the partly cystic, partly solid tumour did not suggest malignancy, while the histological picture revealed malignant transformation of epithelial Brenner islands and invasive growth, and so established the diagnosis

Now, one year and three months after operation the patient is still alive and without abdominal complaints

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of the aorta of cockerels. Neither were they able to find any decrease in the serum cholesterol of the cockerels treated with increasing amounts of nicotinic acid ranging from 0.25 per cent to 1.5 per cent of food.

The present study was carried out in order to investigate the effect of nicotinic acid on cholesterol-fed cockerels which in contrast to rabbits but like man, are omnivorous. Of importance may also be the fact that it has been impossible to induce atherosclerotic changes in the aorta whereas the coronary tree has been involved, too (*Pick et al.* 1952).

Besides nicotinic acid, the effect of a nicotinic acid derivative with protracted resorption, magnesium nicotinate, has also been studied.

## MATERIAL AND METHODS

*Eighty nine one-day-old white Leghorn* cockerels were subdivided *into four* groups. Twelve animals served as controls. They were fed with standard broiler chow. The diet of the second group of 25 birds was supplemented with 2 per cent cholesterol and 5 per cent sesame oil. The third group of 26 cockerels was fed the chow supplemented with cholesterol and sesame oil, and 1 per cent nicotinic acid was added to the diet of this group. The fourth group also including 26 birds received *the same diet as the animals of the third group except that 1 per cent* magnesium nicotinate was given instead of nicotinic acid. The experimental period lasted for twelve weeks. The birds were kept under identical conditions as regards housing, light and temperature. They received food and water *ad libitum*. The food consumption and the weight gain was about the same in all four groups. The birds seemed to thrive on all regimens.

After the experimental period the roosters were killed by decapitation. The thoracic aorta was opened and the aortic lesions were evaluated under a magnifying glass using a grading scale from 0 to 3 according to the principles presented by *Katz & Stamler* (1953). Pieces from aorta, heart muscle and liver were taken, fixed in Bouin's fluid and embedded in paraffin. The sections from aorta were stained with the picro-indigocarmin orcein method (*Romeis* 1948) and the heart and liver sections with the Weigert-van Gieson method or with H & E.

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*Aortic lesions.* According to the colour, the unevenness or the presence of major lesions (the number of the macroscopically discernible atheromas of the aortic wall, the gross examination revealed the following changes.

TABLE 1
*Aortic Lesions*

| | Number of birds | No lesions | 0 to 1 | 1 to 2 | 2 to 3 |
|---|---|---|---|---|---|
| Controls | 12 | 8 | 4 | 0 | 0 |
| Cholesterol | 25 | 0 | 3 | 18 | 4 |
| Cholesterol plus nicotinic acid | 26 | 0 | 16 | 7 | 3 |
| Cholesterol plus magnesium nicotinate | 26 | 1 | 19 | 4 | 2 |

The average gross estimates of aortic lesions were as follows Controls 0 15 cholesterol 1 75 cholesterol plus nicotinic acid 1 17 cholesterol plus magnesium nicotinate 0 96

The results indicate that aortic lesions in the cholesterol fed birds treated with nicotinic acid or magnesium nicotinate tended to be less severe than those of the cholesterol fed birds The incidence of aortic lesions however was about the same in all three experimental groups Spontaneous atherosclerosis although always slight was rather common in our material According to the data presented by *Katz & Stamler* (1953) the incidence of spontaneous atherosclerosis seems to vary rather largely depending probably on the breed in question

Histological examination of the thoracic aorta confirmed the gross findings In the cholesterol fed group intimal thickenings fragmentation of the subintimal elastic fibres and atheromatous changes typical signs of cholesterol induced atherosclerosis were constantly seen In the aortic wall electron microscopically detectable changes typical to atherosclerosis were found in this group too (*Laitinen* to be published) The histological changes were less marked in the two other experimental groups

*Heart* The gross examination of the hearts of the roosters did not reveal any macroscopical visible differences in the four groups except some yellowish plaques in the first few millimeters of the coronary arteries of the cholesterol fed birds This is well in accordance with the results of *Katz & Stamler* (1953)

Microscopically the coronary arteries of the cholesterol fed roosters exhibited a narrow lumen and intimal thickenings

The evaluation of the atheromatous state of the coronary arteries of the two other experimental groups was rather difficult because of the lack of reliable quantitation possibilities The intimal *thickenings and* a narrow lumen however were less frequently observed in coronary arteries of the two groups treated with nicotinic acid derivatives

*Liver* The livers of the animals of the three *experimental* groups were enlarged and greyish yellow in colour Microscopical examination revealed a fairly pronounced mostly central fatty infiltration No differences between the experimental groups were observed in the histological appearance of the liver

## DISCUSSION

Our results indicate that under conditions prevailing in the present experiments nicotinic acid and *magnesium* nicotinate are capable of preventing to some extent cholesterol induced atheromatous changes in the aorta and in the coronary arteries of the cockerel

The [illegible]
three [illegible]
ti n [illegible] [illegible] [illegible] be

tween the cholesterol-fed animals with or without a supplement of nicotinic acid or magnesium nicotinate

The antiatheromatous and suppressing effect of nicotinic acid on elevated cholesterol levels is far from having been made clear *Hardy et al* (1960) observed an increase of cholesterol synthesis from acetate in the rat and in the chick due to nicotinic acid, but contradictory findings have also been reported *Gaylor et al* (1960) were not able to find any excessive degradation of cholesterol to bile acids Referring to these observations *Sinclair* (1960) suggests that nicotinic acid may affect the transport mechanism of cholesterol ending in an opposite effect of the therapeutic aim to decrease the deposition of cholesterol in the intima The present morphological observations are not in agreement with the mechanism claimed by Sinclair (1960), at least not in cholesterol induced atheromatosis of the aorta and of the coronary arteries in cockerels

According to *Berge et al* (1960) a long term treatment of hypercholesteraemia with nicotinic acid in man has often brought about disturbances in the liver function It is conceivable that similar disturbances may have been present in our experimental animals, although related histological changes were lacking

The antiatheromatous effect of nicotinic acid and its derivative seems to be evident not only in the rabbit but also in the cockerel, an animal well suited for atherosclerotic research

Our results differ, however, decidely from the observations of *Hunter & Wong* (1961) In their series of twelve cholesterol-fed cockerels the administration of nicotinic acid failed to induce any hypocholesteraemic effect nor did it prevent the development of aortic atheromatosis On the other hand, *Gaylor et al* (1960) succeeded to depress significantly elevated cholesterol levels in the chick The antiatheromatous effect of nicotinic acid seems thus to be related to its influence on the cholesterol metabolism

## SUMMARY

The effect of nicotinic acid and magnesium nicotinate on experimental cholesterol induced atherosclerosis in cockerels was investigated Both agents seemed to have a preventing effect on the atheromatous changes of the aorta and the coronary arteries as estimated visually and histologically Atheromatous changes were noticeable in the aorta and coronary arteries of the cockerels which received nicotinic acid or its derivate but they were less severe than those of the birds fed with a cholesterol diet only The gross and histological appearance of the liver, a fatty infiltration, was similar in all three experimental groups

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# EFFECT OF EXPERIMENTAL EPIPHYSIOLYSIS ON THE MORPHOLOGY AND FUNCTION OF THE GROWTH ZONE

*By*

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Received 8 v 62

It has been shown that if a fracture (epiphysiolysis) is produced at the junction of the epiphysial cartilage and metaphysis of long bones in growing experimental animals, the formation of hypertrophic cartilage cells continues while, due to cessation of resorption, the hypertrophic portion of the epiphysial cartilage progressively increases in thickness (*Jahn* 1893, *Schmorl & Lossen* 1901, *Dale & Harris* 1958, *Trueta & Amato* 1960, *Westerborn* 1961) The lesion heals within three to eight days through invasion by capillaries and resorption of chondrocytes, with concurrent formation of new bone trabeculae The number of hypertrophic cells may, in certain instances, become abnormally multiplied, and all except the youngest of them exhibit an approximately uniform degree of development and general appearance throughout the zone

Epiphysial cartilage thus thickened presents similarities with that found in rickets This observation has been made by *Schmorl & Lossen* (1901), as well as by *Trueta & Amato* (1960) in their vascular studies on the growth zone

The investigation here reported was designed to elucidate the course of events in the growth zone following separation of the epiphysial cartilage from the metaphysis, and to compare the resulting changes with those observed in rickets To this end, histologic, autoradiographic and microradiographic techniques were employed

## MATERIAL AND METHODS

Young rabbits weighing 300 400 grams were used With the animals under ether anesthesia a longitudinal incision was made through the skin over the distal growth zone of the radius or ulna The periosteum was then divided longitudinally and epiphysiolysis produced with a scalpel at the junction of the epiphysial cartilage and metaphysis Following suture the animals were released without fixation

In the majority of experiments the radius of one extremity and the ulna of the

ether were thus treated For the earliest experiments the radius of one side only was used The contralateral bones invariably served as untreated controls
A total of 65 bones in 40 rabbits were operated on Eighteen animals were injected

6 7 or 8 days after the operation
Immediately after death the distal portion of the radius and ulna on both sides was dissected out and fixed in 10 per cent neutral formalin Decalcification was done in a solution of equal parts of 44 per cent formic acid and 20 per cent mono sodium citrate These procedures were followed by embedding in paraffin For the microradiographic studies specimens from 13 animals were embedded without prior decalcification

... ssible to localize mineral salts in the tissues

## RESULTS

### *Histologic Findings*

Two days after operation the cartilage and metaphysis were seperated by a fracture partially filled with blood and fibrin In the metaphysis adjacent to the fracture coarse trabeculae with thick bone structures covering small basophilic cartilage remnants were observed The cartilage zone had greatly thickened the number of proliferative cells possibly having somewhat increased and the number of hypertrophic cells having greatly increased The latter cells showed a uniform histologic appearance throughout the column The cells nearest to the epiphysiolysis were here and there, somewhat more flattened than normal The cartilage organization was fairly regular and a distinct columnar pattern

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The healing process was initiated by capillary invasion of the cartilage zone from the metaphysis the invasion beginning at different times in different parts of the zone One capillary invaded each cell column and resorbed the cells in an apparently normal manner The resorption proceeded at an increased rate and, in some instances even greatl...

... appeared in the metaphysis The boundaries between these and the aforementioned coarse trabeculae were quite distinct and frequently constituted the sole evidence of the operation (Fig 2) Although healing was initiated at different times in different areas of the growth zone, the total thickness of the cartilage

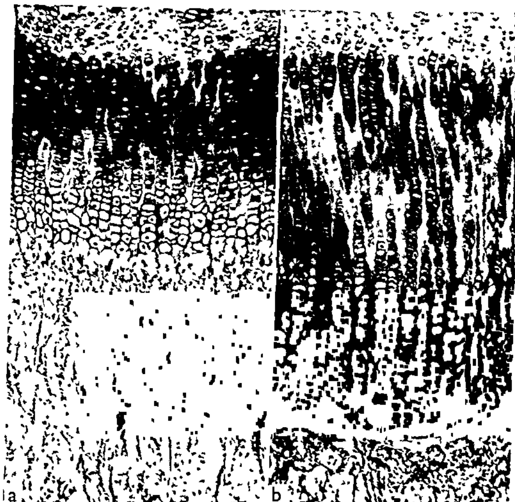



*Fig 1*

Growth zone from distal end of radius Haematoxylin and eosin × 95 a Normal control side b Two days after operation Substantially thickened epiphysial cartilage with increased number of hypertrophic cells Unhealed epiphysiolysis

zone and the new trabeculae was uniform throughout the longitudinal section

In many instances the fracture did not involve the whole of the transverse section at the junction of cartilage and bone When the fracture was located further inside the cartilage zone, signs of cartilage necrosis in the form of cell damage and altered tingibility of the matrix were often observed (*cf Westerborn* 1961) In specimens in which the fracture was, in part, correctly placed, the cartilage zone developed, in the relevant areas, exactly as described above

*Autoradiographic Findings*

*Control Specimens* – The autoradiographic picture was fully consistent with the one previously reported for rabbits (*Engfeldt & Westerborn* 1960) and rats (*Hjertquist* 1961), *i e*,



*Fig 2*

Growth zone from distal end of radius four days after operation Incipient healing of the epiphysiolysis Regular slender trabeculae containing remains of cartilage immediately below the hypertrophic zone Subjacent thereto is a transverse belt of connective and chondro-osseous tissue—the remains of the epiphysiolysis (arrow) Haematoxylin and eosin × 100

jection a radiosulphate uptake with a purely intracellular localization was manifest throughout the cartilage zone Twenty four hours after the injection labelling was observed chiefly at the cell peripheries and in the matrix adjoining the cell columns After the lapse of four or five days the radiosulphate uptake was almost exclusively extracellular in the cartilage zone and by this time labelling of the cartilage remnants in the metaphysial bone trabeculae adjacent to the cartilage zone was also discernible

*Specimens without Signs of Healing* — In these specimens the radiosulphate uptake was fully normal in the greater part of the cartilage zone The rest [illegible]

hypertrophic zone however in contrast to the normal specimens exhibited little or no uptake (Fig 3 a) Similarly, after the lapse of 24 hours a normal uptake pattern was observed except in the part of the hypertrophic zone adjacent to the fissure (Fig 3 b) Certain specimens showed in parts where the cartilage was not so thick an uptake extending as far as the fissure




*Fig 3*

Autoradiograms of growth zone from distal end of radius × 85 a Two hours after radiosulphate injection three days after operation Conspicuous intracellular uptake in the resting cartilage proliferative zone and adjacent part of the hypertrophic zone Little or no uptake in the cartilage containing increased number of hypertrophic cells (the arrows point to the cartilage fracture junction) b Twentyfour hours after radiosulphate injection four days after operation Labelling chiefly of the cell peripheries and pericellular matrix in the resting cartilage zone the proliferative zone and adjacent part of the hypertrophic zone Little or no labelling of cells or matrix in the rest of the hypertrophic cartilage (Arrows as in a) c Four days after radiosulphate injection four days after operation Largely extracellular uptake of $S^{35}$ throughout the thickened cartilage zone (Arrows as in a)

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*Fig 4*

Microradiograms 8 20 Å of undecalcified sections 5 μ thick from distal end of the radius × 85 (a b c) × 55 (d) a Normal Mineralization of the zone of preparat

hypertrophic cartilage




Fig. 4

Four or five days after the injection the specimens showed $S^{35}$ uptake throughout the cartilage zone, the labelling being largely extracellular. In contrast to the findings in the control specimens, no labelling was noted over the trabeculae in the metaphysis. Labelling of the cartilage zone was heavier along the fracture line than elsewhere in the zone (Fig. 3 c). In the control specimens the uptake in the cartilage zone was more uniform.

*Specimens with Partial or Complete Healing.* – Two and 24 hours after the $S^{35}$ injection these specimens showed normal labelling of the entire cartilage zone as far as the metaphysis. In one or two instances, however, no labelling of the juxtametaphysial part of the thickened cartilage was noted. As long as four or five days after the injection normal labelling was found in the cartilage zone, while in the new trabeculae the uptake was seen to extend as far as the junction with the coarse, irregular trabeculae in the metaphysis.

Completion of healing was accompanied by normalization of the autoradiographic findings.

*Microradiographic Findings*

*Control Specimens.* – The growth zones of the untreated control specimens showed identical mineralization patterns. Calcification was observed in the metaphysis at sites corresponding to the trabeculae, the calcification extending to the longitudinal matrix of the epiphysial cartilage between the lowest three or four hypertrophic cells, *i. e.*, the zone of preparatory calcification (Fig. 4 a).

*Specimens without Signs of Healing.* – Substantial deposition of mineral salts was observed in the metaphysis at the sites of trabeculae which, in these specimens, were abnormally coarse and irregular.

Microradiograms from 2 animals sacrificed 2 and 4 days, respectively, after the operation showed no mineralization of the cartilage matrix (except for a minute rectangular area measuring approximately 5 by 8 hypertrophic cells, in the hypertrophic cartilage) (Fig. 4 b).

Specimens from other animals, however, exhibited in the epiphysial cartilage a transverse zone of calcified cartilage matrix parallel with, and a few cells away from the fracture line. The calcified zone had a thickness equivalent to 5–10–30 hypertrophic cells and extended approximately the full width of the epiphysial cartilage (Fig. 4 c). Several specimens exhibited two transverse zones, one of which was contiguous to the fracture. In a few specimens a third transverse calcified zone or irregular calcified areas were also observed higher up in the cartilage and were, as a rule, associated with vessels of varying caliber (Fig. 4 d). In general, only the longitudinal matrix appeared to be calcified.

*Specimens with Partial or Complete Healing.* – Signs of healing were manifest as incipient capillary invasion – one capillary for each cartilage column. At the site of each capillary there was mineralization of



*Fig 5*

the longitudinal cartilage matrix, frequently extending to a level equivalent to 10 12 cells (Fig 5)

When healing was complete the cartilage zone had reassumed its normal mineralization pattern In this group the newly formed trabeculae in the metaphysis showed a normal mineralization pattern

## DISCUSSION

When epiphysiolysis is experimentally produced at the junction of epiphysial cartilage and metaphysis, the hypertrophic cells continue to proliferative, the cartilage becoming progressively thicker as long as the fracture remains open The abnormally thick cartilage thus formed consists largely of hypertrophic cells of normal histologic appearance Within, as a rule, 4–6 days healing becomes manifest in the form of capillary invasion, resorption of hypertrophic cells, and formation of slender trabeculae

Autoradiographic examination was carried out after administration of radiosulphate in order to study the chondroitin sulphate metabolism of the cartilage, previous investigations having shown that this metabolism is reflected in the autoradiographic picture (*Dziewiatkowski* 1951, 1953, *Bostrom* 1953, *Belanger* 1954, *Engfeldt & Westerborn* 1960)

The abnormally thick cartilage formed in these experiments had, to judge by the autoradiographic findings, a normal chondroitin sulphate metabolism in the resting and proliferative zones as well as in the hypertrophic cartilage adjoining the proliferative zone In the remainder of the hypertrophic zone the $S^{35}$ uptake 2 hours and 24 hours after the injection was either substantially diminished or unidentifiable

One possible explanation of this finding is that the cells in that area do not synthesize chondroitin sulphate in the normal way Alternatively, one might postulate a reduced supply of precursors of chondroitin sulphate due to the altered circulatory conditions, the distance to the epiphysial or to the capsular vessels being relatively great It has been shown previously, however, that even very thick cartilage zones are well supplied even though they receive nutrition only from the epiphysial vessels (*Trueta & Amato 1960, Westerborn 1961)* The firstmentioned hypothesis is, therefore, the most plausible The supernumary hypertrophic cells are probably at a stage of development in which little or no synthesis of chondroitin sulphate takes place

The findings here reported are in many respects consistent with observations made during studies of experimental rickets in dogs and rats (*Hjertquist* 1961 a, b) As regards the appearance of the chondrocytes and the stainability of the cartilage matrix no essential difference emerges between the present histologic findings for epiphysial cartilage and those recorded in previous investigations of rickets Furthermore, the autoradiographic findings show conspicuous similarities Two and 24 hours after radiosulphate injection the uptake of $S^{35}$ by rachitic hypertrophic cartilage is limited to the area adjoining the proliferative zone The same is true of the thickened hypertrophic cartilage produced in the present investigation In cases of experimental rickets, radiosulphate labelling throughout the cartilage zone is observed 3 and 7 days after the injection, and in the present investigation 4 days after the injection The distribution of the uptake, moreover, coincides with that described for normal animals

Epiphysial cartilage resembling that associated with rickets has thus been produced by experimental epiphysiolysis This circumstance suggest that neither the accumulation of hypertrophic cartilage cells nor their defective chondroitin sulphate metabolism is, in rickets, pathognomonic for that disease It might also be interpreted as an indication that in rickets the thickening of the epiphysial cartilage and the alteration of its chondroitin sulphate metabolism result from failure of the capillaries to absorb the chondrocytes in the normal manner The results also suggest that the accumulation of hypertrophic cells and the

disturbed chondroitin sulphate metabolism in rickets do not necessarily represent a primary defect of the cartilage cells but may instead be secondary manifestations

The microradiographic studies show that when more than two days have elapsed without signs of healing, a transverse zone of calcified cartilage matrix may be discerned at a distance of three or four cells from the fracture This finding conflicts with the view of *Truela & Amato* (1960) that calcification can take place only in the immediate vicinity of vessels

In the course of healing capillaries penetrate the cartilage columns and at the same time, calcified cartilage matrix appears, frequently extending ten cells or more in advance of the invading capillary, as compared with the normal distance of only two to four cells A possible explanation is that the resorption and ossification which occur are accelerated substantially beyond the normal rate It is conceivable that resorption does not take place until the cartilage matrix has been calcified for a certain time and that accelerated resorption accordingly

prior to invasion of the cartilage

The microradiographic investigation has also demonstrated similarities in the mineralization patterns of rachitic epiphysial cartilage and of the cartilage noted in this study In the present experiments the epiphysial cartilage either showed practically no mineral salts or contained one or more mineralized zones in the matrix of the hypertrophic zone Rachitic hypertrophic cartilage usually is not calcified, though occasionally it has small areas of calcified matrix The healing stages observed after experimental epiphysiolysis may closely resemble those associated with rickets, both in regard to capillary invasion and with respect to deposition of mineral salts (*cf Hjertquist* 1961 b) The microradiographic findings, like the histologic and autoradiographic results thus support the view that the epiphysial cartilage lesions observed in rickets are not pathognomonic of that disease

Neither rachitic, epiphysial cartilage nor the epiphysial cartilage associated with experimental epiphysiolysis exhibits any appreciable capillary invasion of cartilaginous tissues which are devoid of mineral salts During healir [illegible]
physiolysis and in [illegible]
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ible The regular ca[illegible] asion and the resorption of cartilage cells are in both instances, preceded by mineralization of the longitudinal cartilage matrix immediately ahead of the advancing capillaries The arrested resorption of cartilage cells in rickets might therefore be due to the suppression of mineralization in the cartilage matrix

## SUMMARY

Epiphysiolysis of the distal growth zones of the radius and ulna was experimentally produced in young rabbits. The epiphysial cartilage was studied histologically, autoradiographically, and microradiographically two to eight days after the operation. Despite the operative treatment, it continued to proliferate, and a thick cartilage zone was formed The area of the hypertrophic zone which adjoined the fracture synthesized, in contrast to normal cartilage, little or no chondroitin sulphate Microradiograms prior to healing of the epiphysiolysis either showed one or more transverse bands of mineralized cartilage matrix located in the hypertrophic zone or practically no mineral salts in the cartilage

The experimental trauma healed via capillary invasion of the cartilage from the metaphysis This was preceded by mineralization of the longitudinal cartilage matrix just in advance of the invading capillaries The result was a rapid normalization of the growth zone

The above-mentioned three types of examination revealed major similarities between the epiphysial cartilage associated with experimental epiphysiolysis *and that observed in experimental rickets*


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FROM THE DIVISION OF PEDIATRIC PATHOLOGY AT THE DEPARTMENT OF PATHOLOGY AND THE DEPARTMENT OF DIAGNOSTIC RADIOLOGY KAROLINSKA SJUKHUSET STOCKHOLM SWEDEN

# THE ASK UPMARK KIDNEY

## *A Congenital Renal Anomaly Studied by Micro Angiography and Histology*

*By*

ARNE LJUNGQVIST and CURT LAGERGREN

Received 29 x 62

In 1929 *Ask Upmark* described a renal anomaly which he observed in one or both kidneys of 6 patients 5 of whom were also suffering from malignant nephrosclerosis Typical gross features of this anomaly were hypoplasia of the kidney an abnormally small number of pyramids and a deep groove in its surface beneath which a calyx like recess of the renal pelvis terminated without the normal relationship to a pyramid The tissue separating the pelvic recess from the bottom of the groove was fibrotic and apparently contained no cortical or medullary component Microscopically this zone was thyroid like in structure and contained numerous small spherical spaces lined with epithelium and containing colloid like substance there were no signs of inflammation This thyroid like tissue was devoid of glomeruli and contained a large number of thick walled arterial branches Ask Upmark considered the structural alterations to be congenital in origin and possibly due to a disturbance of the interaction between ureterogenic and metanephrogenic anlages during the development of the kidney

Since Ask Upmark published his observations the morpholog...

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## MATERIAL AND METHODS

The two kidneys were removed from women aged 38 and 40 years both on indications of renal hypoplasia and pelvic lithiasis (Fig 1) None of the patients had hypertension

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Fig 1 Fig 2

*Fig 1* Urogram of specimen 1 showing a small kidney with thin parenchyma and a large dilated calyx in the lower pole containing a concrement Centrally a pelvic recess with a dilated tip is seen

*Fig 2* Frontal section through specimen 1 showing the deep groove with the underlying cystically dilated pelvic recess which divides the kidney into one cranial and one caudal pole both of which consist of only a few pyramids There are calculi in the renal pelvis

graphed by the method described in an earlier paper (*Ljungqvist & Lagergren* 1962) For histologic examination these blocks were re embedded and serial sections were cut from the block of each kidney that comprised the groove pelvic recess and surrounding tissue From each of the remaining blocks 3 sections were cut The sections were stained with haematoxylin eosin or by van Gieson's method in some sections after pre staining for elastic fibres according to Verhoeff A few sections were stained for fibrinoid substance by Ladewig's method

*Gross Findings*

*Specimen I* (Fig 2) The kidney measured 9 by 5 by 3 5 and was abnormal in its configuration Both poles were bulky A deep groove passed centrally across the lateral aspect of the kidney The renal pelvis contained friable and greyish white calculus material in the lower pole adherent to the wall of the pelvis which at this point was

beneath which it

recess bisected the

There was normal

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practically identical with that of specimen 1 but the poles were not as bulky A deep horizontal groove passed centrally across the surface of the lateral aspect and a zone of fibrous tissue separated its bottom from the end of a pelvic recess The latter divided the kidney into two parts the lower of which had 3 pyramids and the upper



Fig 3 A



Fig 3 B

*Fig 3 A* Micro angiogram from the cortical zone of a normal kidney showing an interlobular artery which gives off arterioles One on the left forms a glomerular tuft and the one on the right is thin and ends blindly × 25

*Fig 3 B* Histologic section of the area depicted in Fig 3 A The blindly ending arteriole leads to the vascular pole of a degenerated glomerulus *Htx* × 90

2 The renal pelvis contained a single coral stone but the cystically dilated end of the recess was free from calculi

*Histologic Findings*

*Specimen 1*—The tissue separating the pelvic recess from the bottom of the groove had a thyroid like structure with numerous small spherical spaces lined with a single layer of epithelium and containing a colloid like substance (Figs 5B and 6B)

matory cells

tians and in the lumina of the tubules there were streaks of lymphocytes and plasma cells arranged fanwise In these areas there was atrophy of the tubules a slight thickening of the walls of the pre glomerular vessels due to cellular hyperplasia and periglomerular fibrosis many of the glomeruli were totally fibrotic There was no evidence of the thyroid like structure in these areas Where the renal tissue was unchanged the arteries too were normal



Fig 4 A   Fig 4 B

Fig 4 A Micro angiogram from the juxtamedullary zone of a normal kidney showing an aglomerular arteriole which divides (*bottom right*) into arteriolae rectae of the medulla × 25

Fig 4 B Histologic section of the area indicated in Fig 4 A The arteriole passes through a degenerated glomerulus the fibrotic capsule of which is clearly seen *van Gieson* × 90

*Specimen 2* As in specimen 1 the tissue separating the pelvic recess from the bottom of the groove was thyroid like in structure There were densely disposed spaces containing a colloid like substance (Fig 5 B) and many scarred glomeruli with a patent capillary loop in the vascular pole The pelvic recess was lined with transitional epithelium and the subepithelial tissue was sclerotic and slightly infiltrated by lymphocytes There were no signs of pyelonephritis or nephrosclerosis

*Micro angiographic Findings*

The principal features of the micro angiograms were practically identical in the two kidneys The vascular pattern of the thyroid like tissue between the pelvic recess and the groove was that of an abnormally small renal lobe with the cortex and medulla clearly outlined (Fig 5 A) The interlobular arteries were tortuous and thin and gave off a large number of branches of capillary calibre which formed networks of looping vessels (Fig 6 A) Serial histologic sections showed these networks to consist of afferent and efferent arterioles communicating through the vascular pole of degenerated glomeruli (Fig 6 B) and of anastomosing capillaries arising from the efferent segments of these aglomerular arterioles and surrounding the colloid filled spaces The medullary vessels originated in the juxtamedullary zone they were all ramifications of aglomerular arterioles and thus were of the arteriolae rectae verae type (Fig 5 A) They extended to the wall of the pelvic recess In the rest of specimen 2 the vascular pattern was normal for the age but in the inflamed parts of specimen 1 it was typical of chronic pyelonephritis (*Lagergren & Ljungqvist* 1962)



*Fig 5 A*



*Fig 5 B*

*Fig 5 A*

*Fig 5 B* Histologic section of the area depicted in Fig 5 A The area has a thyroid-like structure and no pattern of a renal lobe is evident *van Gieson* × 25

## DISCUSSION

The gross picture of the two kidneys bore a close resemblance to that described by *Ask-Upmark* in 1929 The histologic appearance also corresponded essentially with his description apart from the absence of nephrosclerosis and the presence of scarred glomeruli in areas of thyroid-like structure From his gross and histologic findings Ask-Upmark concluded that the pelvic recess was unrelated to any pyramid In the present specimens, too, these methods suggested that the thyroid-like structure was not that of a renal lobe, the vascular pattern visualized by micro angiography, on the other hand, was indisputably that of a renal lobe, for there were well-defined though altered cortical and medullary vessels and a distinct cortico-medullary border

The histologically most characteristic feature of the affected tissue was its thyroid like structure It is generally held that the presence of such tissue in the kidney is a result of chronic pyelonephritis, but it has also been interpreted as a congenital malformation of the renal tubules (*Fahr* 1938, *Voth* 1949) In the present material the positional relation-





Fig 6 A    Fig 6 B

*Fig 6 A* Micro angiogram of the cortical zone of the hypoplastic lobe of specimen 1 There is a network of aglomerular anastomosing vessels forming loops and kinks × 60

*Fig 6 B* Histologic section from part of the area depicted in Fig 6 A One vessel is seen to pass through the vascular pole of a scarred glomerulus *van Gieson* × 120

ship between the spaces containing colloid-like substance and the cortical capillaries suggests that the former were dilated tubules On the basis of the findings of a previous investigation (*Lagergren & Ljungqvist* 1962) it was tentatively concluded that such dilatation of the tubules observed in chronic pyelonephritis may be accounted for by one of the following two factors (i) pyelonephritic damage to normal renal tissue and (ii) pyelonephritic damage to ectopic juxtamedullary tissue in the cortex In the latter event, as was the case in the thyroid-like regions of the kidneys of the present study, the afferent and efferent arterioles were in communication *via* degenerated glomeruli throughout the cortex Glomerular degeneration will normally give rise to such aglomerular arterioles only in the juxtamedullary zone (Fig 4), while further out in the cortex the afferent arterioles end blindly at the vascular pole of the degenerated glomeruli (Fig 3) (*Ljungqvist & Lagergren* 1962) In both specimens of the present study the vascular alterations in the hypoplastic lobe accompanying glomerular degeneration suggested the whole of its cortex to be juxtamedullary in type, which would most likely be a congenital malformation The thyroid-like struc-

ture may be ascribed to persistant function of the degenerating glomeruli owing to the maintainance of blood flow through them As in pyelonephritic scarring (*Kleeman et al* 1960) the marked fibrosis of the pelvic wall and adjacent part of the medulla may have impeded the outflow from the tubules with consequent obstruction and dilatation

The fact that the cortex of the hypoplastic lobe was composed entirely of juxtamedullary nephrons may well be due to a disturbance of the anlage of the lobe and degeneration of the nephrons would account for its thyroid like structure While the cause of this degeneration is obscure it is probable that the malformed part of the kidney would be unusually susceptible to noxa and that the degeneration would therefore occur early in life This theory is supported by the fact that 5 out of 6 patients in Ask Upmarks series were adolescents

That the microscopic changes point to a congenital malformation of the renal tissue and that the abnormal number of pyramids and the pelvic recess are unlikely to be of post natal origin constitute compelling evidence that the Ask Upmark kidney is a congenital anomaly

## SUMMARY

Micro angiographic and histologic studies were carried out on 2 Ask Upmark kidneys removed from female patients aged 38 and 40 years

... bottom of the groove was thyroid like in structure and micro angiographic examination showed it to be a hypoplastic renal lobe with the cortex and medulla clearly demarcated Examination of the blood vessels revealed that the cortex of this area consisted entirely of juxta medullary nephrons This feature was regarded as a congenital anomaly as were the abnormal number of pyramids and the pelvic recess

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The fact that the cortex of the hypoplastic lobe was composed entirely of juxtamedullary nephrons may well be due to a disturbance of the anlage of the lobe, and degeneration of the nephrons would account for its thyroid like structure. While the cause of this degeneration is obscure, it is probable that the malformed part of the kidney would be unusually susceptible to noxa and that the degeneration would therefore occur early in life. This theory is supported by the fact that 5 out of 6 patients in Ask-Upmarks series were adolescents.

That the microscopic changes point to a congenital malformation of the renal tissue and that the abnormal number of pyramids and the pelvic recess are unlikely to be of post-natal origin constitute compelling evidence that the Ask-Upmark kidney is a congenital anomaly.

## SUMMARY

Micro-angiographic and histologic studies were carried out on 2 Ask-Upmark kidneys removed from female patients, aged 38 and 40 years. Both kidneys showed hypoplasia, an abnormally small number of pyramids and a deep transverse groove in the surface. A long pelvic recess extended towards the groove. The tissue separating the end of the recess from the bottom of the groove was thyroid like in structure and micro-angiographic examination showed it to be a hypoplastic renal lobe with the cortex and medulla clearly demarcated. Examination of the blood vessels revealed that the cortex of this area consisted entirely of juxtamedullary nephrons. This feature was regarded as a congenital anomaly as were the abnormal number of pyramids and the pelvic recess.

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# THE SIGNIFICANCE OF HISTOLOGICAL DIAGNOSIS IN GLANDULAR TOXOPLASMOSIS

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The significance of a histological diagnosis in glandular form of toxoplasmosis was first stressed simultaneously by *Saxen et al* (1958) in Finland and by *Piringer-Kuchinka et al* (1958) in Germany The main features of the histological changes found in cases previously diagnosed by serological methods had already been described by *Gard & Magnusson* (1950) and by *Siim* (1950) These changes have been discussed recently by a number of authors (*Roth & Piekarski* 1959, *Saxen & Saxen* 1959, *Budzilowitch* 1961, *Siim* 1961, *Stansfeld* 1961) However, the significance of this picture in the diagnosis of glandular toxoplasmosis needs further elucidation based upon more ample series The present paper relates to a new series of 50 patients, clinically unsuspected to toxoplasmosis, and tested serologically following a tentative histological diagnosis

## THE CASES

After a presentation of the characteristic changes in glandular toxoplasmosis made before the Finnish Association of Pathology in 1957, pathologist in Finland kept a watchful eye on the occurrence of this disease in their daily routine Consequently, during the years 1958–61 they diagnosed a number of cases as being suggestive of glandular toxoplasmosis [1] The present authors have, with no fore knowledge of the clinical data of the patients, examined all these slides, and found 82 of them to fulfil the criteria for toxoplasmosis presented in our earlier papers Of these cases, 50 had been subsequently tested serologically, and only these cases are presented below It hardly needs stressing that no cases have been subsequently excluded on the acquirement of knowledge of the clinical data and the results of the serotests

The present paper lays stress only on the histological features in glandular toxoplasmosis, and their diagnostic significance The clinical data will be presented in detail later

1 The authors are indebted to the heads of the pathological laboratories in Finland Dr L Hjelt prof O Jarvi dr L Meurman prof C von Numers prof K Setälä prof H Teir and prof U Uotila



*Fig 1*

A low power view showing a diffuse collection of pale epitheloid like cells and large germinal centres with indistinct borders Htx—van Gieson × 60

The original diagnoses were made on slides stained with haematoxylin-van Gieson or haemalaun-eosin In addition, the following staining methods were used in subsequent studies: Periodic acid Schiff, Berlin blue for blood pigment, Feulgens reaction, Weigert's stain for fibrin, different reticulin stains, Ladewig-procedure etc

RESULTS

*Histology*

In all cases, the histological changes are quite uniform, as the series had been selected on the basis mentioned above The most typical changes in our cases are as follows·

1 The follicular pattern is preserved, but owing to a diffuse or focal collection of large, pale epithelioid like cells, the margins of the follicles are not distinct (Fig 1)

2 The large epitheloid like cells show indistinct borders, their cytoplasm is faintly eosinophilic, and small vacuoles are often seen The nucleus is vesicular and only slightly stainable with Feulgens reaction Mitoses have not been noted in these cells (Figs 2 and 3)

3 A strong proliferation of large reticulum cells is always noted in the germinal centres Mitotic figures are common, and many of the cells contain an abundance of nuclear debris,p a S -positive globules and bas-

*Fig 2*

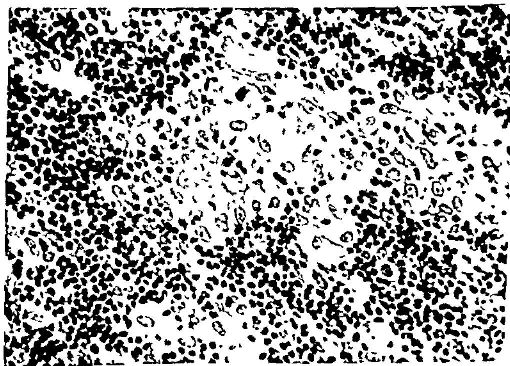


*Fig 3*

*Figs 2 and 3*

Focal and diffuse collection of epitheloid like cells with indistinct cells borders and vesicular nuclei Htx—van Gieson × 300 and 600

*Fig 4*

*Fig 5*

*Figs 4 and 5*

Strong proliferation in the germinal centres of large *reticulum cells containing nu*clear debris in abundance Htx—van Gieson × 300 and 600

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*Fig 3*

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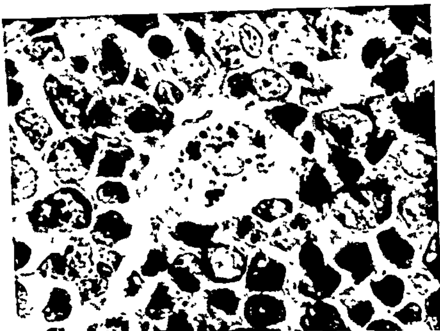


*Fig 8*

A large reticulum cell in a germinal center containing nuclear debris and p a S positive coarse granules clearly distinguishable from the Toxoplasma like bodies in Fig 8 Htx—p a S × 1500

Dye test

| Complement fixation | <1 4 | 1 4 | 1 16 | 1 64 | 1 256 | 1 1024 | 1 4096 | 1 16384 | 1 65536 |
|---|---|---|---|---|---|---|---|---|---|
| <1 4 | •• | | | •• | •••• | | •• | • | |
| 1 4 | | | | | •• | • | ••••• | •• | |
| 1 8 | | | | | •• | •• | ••• | ••• | |
| 1 16 | | | | •• | •• | •••• | • | ••••• | • |
| 1 32 | | | | • | | ••••••••• | ••••• | ••• | •• |
| 1 64 | | | | | • | •• | | | |
| 1 128 | | | | | | | • | | |

*Fig 9*

Sabin Feldman "Dye test" titers and complement fixation test titers in the present material and in the 22 cases previously reported by us (Saxén and Saxén 1959)

*Fig 6*
A sinus filled with pleomorphic mononuclear cells. Htx—eos × 600

*Fig 7*
Two epitheloid like cells containing p a S-positive ovoid particles resembling Toxoplasma organisms Htx—p a S × 1500

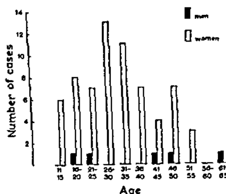



*Fig 11*

The age and sex distribution of the 72 cases of glandular toxoplasmosis

served at the other end of the body These p a S -positive granula often formed small microcysts in the vicinity of the nucleus, and they are easily distinguishable from the coarse granules found in the large cells of the germinal centres The authors are inclined to interpret these as true toxoplasms (Figs 7 and 8)

*Serology*

The serological tests were made in the Department of Virology, University of Helsinki, the State Serum Institute, Helsinki, and the Department of Medical Microbiology, University of Turku The distribution of the titres in the serotests are indicated in Fig 9 The diagram gives the titres of the 48 new cases and, in addition, those of the 22 cases previously reported but collected in a similar manner (If several tests were made from the same patient the highest values are indicated Two cases with high CF titres are included in the material but excluded from the table since the dye test was not performed)

In addition, some typical cases are presented in Fig 10

*Age and sex distribution*

As was the case in our earlier series, most of the patients were young adults, with a definite predominance of women The distribution is given in Fig 11

*Frequency*

The material collected on the basis of histological diagnoses gives us no reliable figures for the true frequency of glandular toxoplasmosis, as very many of the cases are obviously correlated with only slight clinical symptoms not leading to biopsy Nevertheless, a relative incidence of this disease in the pathologist's practice can be of certain

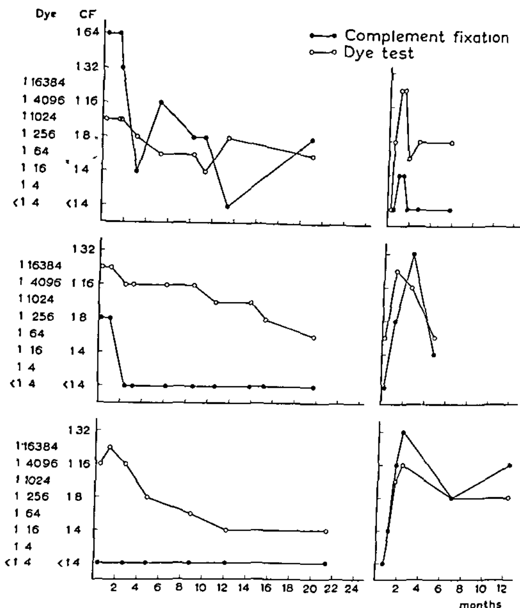



*Fig 10*

Examples of cases in which the DT and CF titers were examined repeatedly after the biopsy (point zero day of biopsy)

ophilic particles Fibrin is often encountered between the cells, and occasionally extravasation of red blood cells is present (Figs 4 and 5)

4 In many areas, the sinuses are filled with large mononuclear cells The size of their nuclei varies, and the nucleoli are prominent (Fig 6)

5 Necrosis, Langhans-giant cells, Reed-Sternberg-like cells and eosinophils are seen but seldom, but plasma cells may be more numerous

In some slides, stained with p a S, a fine-dispersed granulation was noted in the cytoplasm of the epitheloid cells The granula were of equal sizes, fusiform or sickle-shaped, and often a clear vacuole was ob-

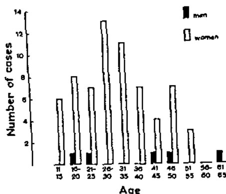



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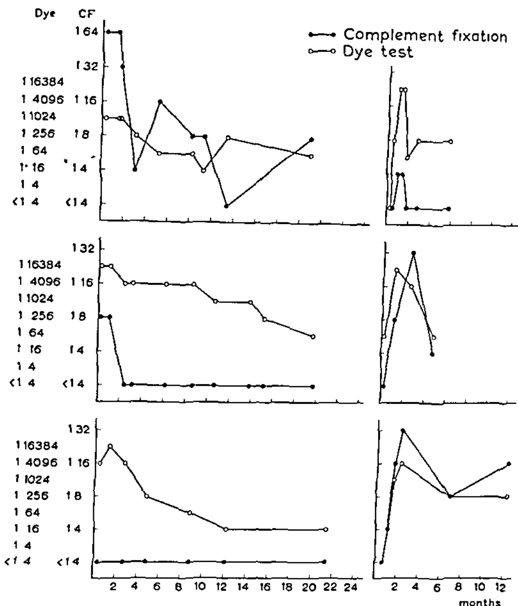



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granulae interpreted as toxoplasms. It may be added that in some cases of abundant nuclear debris and other changes which are imitative of toxoplasmosis we have been able to isolate adenovirus from the lymph nodes (through the courtesy of *Dr Kari Penttinen*).

One of the cases in our basic series later proved to be a malignant lymphogranulomatosis. This example demonstrates the most important misinterpretation in the diagnosis of glandular toxoplasmosis. In view of the marked cellular pleomorphism sometimes seen in toxoplasmosis it is quite probable that some of the cured cases of Hodgkins diseases in fact have been cases of glandular toxoplasmosis. It is by no means an easy task to make a differential diagnosis of glandular toxoplasmosis and an initial stage of Hodgkins disease. Nuclear pleomorphism and high mitotic activity are hardly applicable criteria, typical large histiocytes can be very scanty in some cases of toxoplasmosis and the general signs of inflammation can be slight. However, eosinophilic cells and cell types resembling Reed-Sternbergs cells are extremely rare in glandular toxoplasmosis and should arouse the pathologist's suspicion. In cases termed benign Hodgkins disease or Hodgkins paragranuloma differential diagnosis may be even more difficult (see *Jackson & Parker* 1944, *Harrison* 1952, *Saxén & Saxén* 1959).

## SUMMARY

A report is given on a series of 72 cases of glandular toxoplasmosis. In all cases the diagnosis was first suggested by a pathologist on the basis of changes in lymph node biopsies from patients whose clinical examination had not indicated this disease. Subsequent serotests were positive to a high percentage (more than 90). The microscopical changes characteristic of the glandular form of acquired toxoplasmosis are described and the differential diagnosis is discussed. Toxoplasma-like bodies are described in some lymph nodes.

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interest, *Turunen* (to be published) has collected the lymph node biopsies studied in our pathological laboratory (*E. & L. Saxén*) during the period 1958–60. Table 1 indicates some of the most important groups of diagnoses made in these 1000 lymph node biopsies.

TABLE 1

*The Frequency of Certain Findings in 1000 Lymph Node Biopsies Histologically Examined by E. and L. Saxén 1958–60. Compiled by M. Turunen (to be published)*

| | *Men* | *Women* | *Total* |
|---|---|---|---|
| *Toxoplasmosis* | *1* | *21* | *22* |
| Tuberculosis | 111 | 178 | 289 |
| Other specific inflammations | 16 | 16 | 32 |
| Non-specific inflammations | 162 | 129 | 291 |
| Malignant lymphogranulomatosis | 26 | 14 | 40 |
| Other malignant lymphomas | 30 | 22 | 52 |
| Metastatic tumours | 74 | 66 | 140 |

## DISCUSSION

The investigation was based on a series selected on histological basis alone, and from a patient population clinically unsuspected of toxoplasmosis. The results show a definite correlation between the "toxoplasmic" histological changes in the lymph gland and high titres in toxoplasma serotests. The titres in our series are definitely higher than those in a control series with corresponding age distribution. *Grönroos* (1955) compiled data from such a series in Finland, and his results obtained from the control group of healthy young adults show that sera with positive CF-titers are obtained in 13.3 per cent and sera with DT titers above 1/256 in 22.7 per cent. Furthermore, the examples indicated in Fig. 10 show that in at least a large proportion of cases in the present series, a rapid increase in the titres followed the first symptoms leading to biopsy, followed by a relatively slow decrease in the values. This might be considered as evidence for a recent toxoplasma infection. Accordingly, although we have not as yet isolated the micro-organisms from the lymph nodes, we are inclined to feel that in most, if not in all of our cases, a true acquired toxoplasmis was the cause of the histological changes. Thus the histological picture is diagnostic at least in the same degree as in some other specific infections, *e.g.* tuberculosis.

As regards the differential diagnosis, some remarks are necessary, although in the main we should refer to our earlier discussion (*Saxén & Saxén* 1959). The histological characteristics presented above (large epitheloid like cells, pseudogranulomas, abundance of nuclear debris, hyperplasia of the Flemming's centres, fibrin etc.) are all rather common features in inflammatory reactions of the lymph nodes. However, when one keeps to *all* the criteria presented, the diagnosis seems to be possible even in the absence of microcysts and the p.a.S.-positive

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A report is given on a series of 72 cases of glandular toxoplasmosis In all cases, the diagnosis was first suggested by a pathologist on the basis of changes in lymph node biopsies from patients whose clinical examination had not indicated this disease Subsequent serotests were positive to a high percentage (more than 90) The microscopical changes characteristic of the glandular form of acquired toxoplasmosis are described and the differential diagnosis is discussed Toxoplasma like bodies are described in some lymph nodes

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6 [illegible]

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THE DEPARTMENT OF OTOLARYNGOLOGY AND THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY UNIVERSITY OF LUND LUND SWEDEN

# CHEMICAL AND SEROLOGICAL ANALYSIS OF ANTIGEN PREPARATIONS FROM STAPHYLOCOCCUS AUREUS

## *A Comparison Between the Products Obtained by Verwey s and Jensen s Techniques*

*By*

THORVALD LOFKVIST and JOHN SJOQUIST[1]

Received 4 v 62

In 1940 *Verwey* (1) described a method for preparation of antigens from staphylococci Using a strain of type A as described by *Julianelle & Wieghard* (2) Verwey prepared five different fractions, one of which (fraction B) was a type specific antigen of a protein nature To the present time, no further reports concerning the chemical or serological nature of this antigen have appeared

*Jensen* carried out an analysis of staphylococcal antigens in 1959 (3 4) and found three common antigens for coagulase positive strains An extensive study made of one of these antigens (antigen A) indicated that it was present in large amounts in a Cowan type I strain By the use of gel precipitation technique antibodies against this antigen were generally found in normal human sera Studies by Jensen suggested the antigen was a previously unknown polysaccharide

*The present* studies were undertaken to characterize more fully the chemical nature of the antigen described by Jensen A comparative study between Jensen s antigen and an antigen sample prepared from the same bacterial strain by Verwey s technique (fraction B) is also reported

## MATERIAL AND METHODS

*Strain* A staphylococcal strain type Cowan I phage type 52 and 52 52 A/79 80 by Routine Test Dilution (RTD) and 1000 times RTD respectively rich in antigen A

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We wish to acknowledge our indebtn
f Bacteriology University of L
at our disposal and for construc
quist The Institute of Bacteriol

9 *Saxen E Saxen L & Gronroos P* Glandular toxoplasmosis A report of 23 histologically diagnosed cases Acta path et microbiol scandinav 44 319 328 1958
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*Serolog cal methods* Titration experiments and tests in connection with electro phoresis of antigen preparations were carried out by precipitation in *gel* using a 2 per cent agar (Reinagar Behringwerke) buffered with sodium veronal (ionic strength = 0 05 pH 8 2) Melted agar gel (2 5 ml) was poured onto a microscope slide (7 6 × 2 6 cm) and immediately enclosed in a humid chamber at room tempe rature After the gel had set a rectangular through (4 5 × 0 1 cm) was cut out in the middle of the agar Two rows of circular basins (diameter 0 2 cm) were made on both sides of the trough at a distance of 0 3 cm from it Antiserum (30 µl) was added to the trough and 5 µl of the antigen sample was pipetted into each of the basins (Titrating antiserum 30 µl of an antigen solution was added to the trough and 5 µl of the antiserum to the basins) After incubation in the humid chamber for 24 hours the agar slides were eluted in saline for one day and washed in distilled water for one to two hours Afterwards they were covered with filter paper and dried at room temperature overnight Subsequently the slides were stained in a solution of Amido black (Amido black 10 B 17 g glacial acetic acid 10 ml ethanol 1250 ml distilled water 3850 ml) for ten minutes after which the excess dye was r nsed off in a bath of the f llowing composition glacial acetic acid 10 ml ethanol 1250 ml distilled water 3850 ml In experiments for reaction of identity the agar diffus on technique described by *Ouchterlony* (7) was used with the modification that 2 per cent agar slides of 7 6 × 2 6 cm with circular basins of 0 2 cm filled up w th 5 µl of reactants were used Confirmatory examinations in this respect were per formed according to the method of *Walsworth* (8) using matrix apertures at the agar surface of 0 2 cm loaded with 20 µl of reactants The agar slides used in the two methods for reaction of identity were stained in a similar manner as des cr bed above

Precipitation in *tubes* was performed for subsequent chemical analysis of the

... e e colle ted for *chemical analysis* and the super natants tested for excess of antibodies against antigen A Controls (negative) were performed using a sample prepared by Jensen s method from a strain lacking an tigen A Th s strain was obtained from Dr Klaus Jensen and designated by him as strain # 14

*Chemical methods* Protein was determ ... r the *Waldell* (10) method Prep ... acids by paper chromatography ... r lysis in 6 N HCl for 24 hours Th ... gram by d pping in 0 02 per cent ... acetone (11) followed by drying at 50° C Ultra v olet spectra of aqueous sol ... ent preparations were ... on with cysteine and ... ent and disappearance ... the anthrone reaction ... ose and mannose as standards hexosam ...

Examinations of purines and pyr midines were carried out by paper chromato graphy in iso propanol 2 N hydrochloric acid (65 35) on samples hydrolyzed with 0 1 N HCl at 100° C for 1 and 24 hours respectively The spots were located on the chromatograms by means of a fluorescent screen (16) Adenine and guanine were used as controls

Jensen s antigen A preparation was d gested with trypsin (Novo Copenhagen) in a pH stat at pH 7 6 and 37° C

was obtained through the kindness of Dr Klaus Jensen Copenhagen and was used throughout these investigations

*Cultivation technique* The organisms were grown on agar plates (diameter 13 cm) of the following composition 1 liter of tap water 5 g of meat extract[1] 3 g of NaCl 2 g of $Na_2HPO_4 2H_2O$ 1 g of glucose 10 g of peptone 11 g of agar, pH 7 8 —The bacteria were harvested after 18 hours' growth at 37° C Afterwards they were washed three times in saline before the fractionation procedures

*Fractionation Methods*

*A Antigen Fractionation as Described by K Jensen*

*Some portions of purified antigen preparation* were courteously supplied by Dr Jensen and used for comparative experiments

In our production of purified antigen Jensen s technique was followed in all essentials Washed bacteria from 100 agar plates were suspended in 500 ml of phosphate buffer (9 parts 1/15 M $KH_2PO_4$ + 1 part 1/15 M $Na_2HPO_4$ pH 5 9) and boiled for 1 hour followed by rapid cooling to + 4° C and centrifugation at 1000 x g for 3 hours at + 4° C (*Subsequent centrifugations were always performed at 1000 x g and +4° C)* The supernatant was adjusted to pH 3 0 with 0 1 N HCl and the precipitate formed was removed by centrifugation for 1 hour and suspended in phosphate buffer pH 5 9 To the clear solution was added ethanol to a final concentration of 70 per cent and the resultant precipitate was spun down and dissolved in phosphate buffer pH 5 9 Trichloroacetic acid was then added to a final concentration of 2 per cent and the precipitate was collected by centrifugation and again dissolved in phosphate buffer Subsequently the antigen was precipitated by adding ethanol to a concentration of 70 per cent and collected by centrifugation The precipitate was dissolved in phosphate buffer and reprecipitated by ethanol The ethanol precipitation step was then repeated three times The final precipitate was dissolved in distilled water and dialyzed against distilled water for 2 days The solution was afterwards lyophilized and stored at —20° C The product obtained is referred to as Jensen s antigen A preparation

*B Preparative Electrophoresis of Jensen s Antigen A Preparation*

The final antigen A preparation obtained by Jensen's method (150 mg) was dissolved in 1 ml of 0 05 M $(NH_4)_2CO_3$ pH 8 9 and applied to a Pevikon block- 40 × 8 5 × 0 5 cm for electrophoresis in 0 05 M $(NH_4)_2CO_3$ pH 8 9 (5) The electrophoresis was run for 24 hours with a voltage setting of 300 V (40mA) Pieces of Pevikon 1 cm in width were cut out perpendicular to the length of the block and each piece was eluted with distilled water The extracts were read at 260 and 280 mμ and tested for serological activity in gel

*C Antigen Fractionation as Described by Verwey*

Following Verwey s preparation scheme the washed bacteria from 100 agar plates were disintegrated in a *Hughes* press (C) without abrasive at —40° C *The disrupted* cells were suspended in 500 ml of distilled water and centrifuged for 1 hour at + 4° C and 100 000 × g The clear supernatant was adjusted to pH 3 5 with 0 1 N HCl and the precipitate formed was spun down for 1 hour at + 4° C and 1000 × g Trichloroacetic acid (50 per cent) was added to the supernatant to a final concentration of 12 5 per cent and the resultant precipitate was collected by centrifugation for 1 hour at + 4° C and 1000 × g and dissolved in distilled water The solution was then adjusted to pH 7 5 with 0 1 N NaOH and dialyzed against distilled water for two days A slight flocculation appeared during dialysis which was removed by centrifugation at 100 000 × g The supernatant was lyophilized and kept at —20° C This fraction corresponds to the antigen protein( fraction B) according to Verwey

*Sera* Serum from one of the authors (T I ) was used as antiserum unless otherwise stated Merthiolate (1 10 000) was added to the serum as preservative

---

1 Meat extract nr ... Sweden
- Pevikon C 870 = ... polyvinylchloride manufactured by Stockho... m Sweden

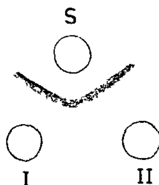



*Fig 2*

Reaction of identity" between the antigen A prepared by Jensen (I) and that prepared by us (II) S = serum T L A modified Ouchterlony technique was used (see text)

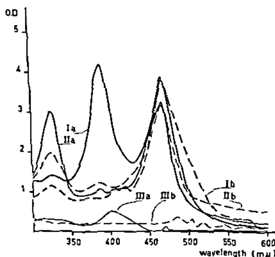



*Fig 3*

barbituric acid assay (15) was less than 0 15 per cent Hexosamines were less than 0 5 per cent whereas hexuronic acids could not be detected

For further characterization precipitation of the antigen with antiserum was carried out in tubes with an excess of antibody (By precipi-

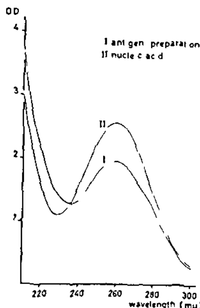



*Fig 1*

UV-spectrum of Jensen s antigen preparation in an aqueous solution The spectrum for a solution of nucleic acids is shown for comparison

## RESULTS

### *Characterization of the Product after Fractionation by Jensen's Method*

An antigen sample supplied by Dr Klaus Jensen gave a titer of 1 16 vs antiserum (T L ) using the agar gel precipitation technique described above starting from a concentration of 10 mg/ml The protein content of this sample was approximately 30 per cent whereas the hexose content was 4 per cent Ultra violet spectrum of the product dissolved in water (Fig 1) showed a strong absorption at 260 mμ, suggesting a high content of nucleic acids These findings do not confirm Jensen's suggestion that antigen A is a polysaccharide (4) The product prepared in our laboratory using Jensen's procedure contained 60 per cent protein and 2 per cent hexoses The ultra-violet spectrum was identical with that for the sample obtained from Jensen The titer of our preparation with the antiserum was 1 32 using a primary concentration of 10 mg ml Our sample also showed "reaction of identity" with Jensen's product as can be seen in Fig 2 This figure also shows the same heavy precipitate zone as seen in Jensen's pictures (4) Commenting on this feature of the precipitates *Oeding* (17) suggested the possibility of two or more separate systems being present

To obtain more detailed information about the carbohydrate composition of the antigen preparation, Dische s general cysteine-sulfuric acid reaction was carried out Fig 3 gives the results of this experiment (for detail, see legend to the figure) These curves show that the only class of saccharides present was pentoses, which yield a maximum at 390 mμ in this reaction, which disappears upon the addition of water

The sialic acid content calculated from equation 2 in Warren s thio-

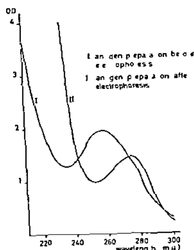



*Fig 5*

UV spectrum of the antigen preparation before and after purification by electrophoresis on Pevikon block.

### *Further Purification of the Antigen Preparation*

Antigen A migrated slowly towards the anode in electrophoresis at pH 8 9 on a Pevikon block Fig 5 gives the ultra violet absorption curve for the antigen active fraction recovered after electrophoresis For comparison a spectrum of an antigen preparation recorded prior to electrophoresis is included Clearly the antigen preparation has been freed from most of its nucleic acid contaminants A negative finding for purines and pyrimidines on paper chromatography of a hydrolysate verified this results In addition an examination of the purified sample using Dische s general cysteine sulfuric acid reaction indicated the absence of any sugar moieties Paper chromatography for amino acids of an acid hydrolysate showed most of the amino acids ordinarily found in natural proteins

Antibodies against the purified antigen preparation were found in all 25 of the normal human sera tested by agar gel precipitation "Reaction of identity between the sample purified by electrophoresis and the one obtained from K Jensen is shown in Fig 6 The figure also shows that the preparations contain more than one antigen

### *Relationship between the Products Obtained by Verwey s and Jensen s Methods*

The antigen preparation fractionated by Verwey s method gave precipitation in gel with the antiserum (T L ) used in the precipitation studies for Jensen s antigen sample Furthermore the antigen fraction (Verwey) was precipitated by all 25 normal human sera tested in agar

for hexoses, hexosamines and sialic acids The figures given in Table I, expressed as percentage of protein content, seem to be too low to originate from a polysaccharide antigen Probably they represent part of the antibody (18)

TABLE I

*Analysis of the Antigen-Antibody Precipitates Figures for Carbohydrates Expressed as Percentages of the Protein Content*

| Protein in mg | Sialic acids in % | Hexosamines in % | Hexoses in % |
|---|---|---|---|
| 0 384 | 1 4 | 2 8 | 7 2 |
| 0 568 | 1 0 | 3 5 | 7 4 |

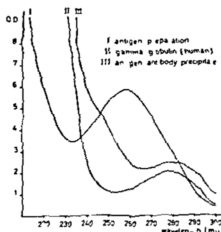


*Fig 4*

UV spectrum of solutions of the antigen A antibody precipitate the antigen A preparation and human gamma globulin

Examination of a hydrolysate of the antigen-antibody precipitate by paper chromatography revealed no nucleic acids Recordings of the UV spectrum after dissolving the antigen antibody precipitates were also taken The precipitates were dissolved in 0 1 N NaOH and subsequently neutralized with 0 1 N HCl before dilution with saline Fig 4 shows the result of this experiment For comparison the curves for the antigen preparation and for a normal human gamma globulin preparation are included Obviously, the curve of the antigen antibody precipitate is of the same character as that of the gamma globulin Thus, the maximum at 260 mμ which is characteristic for the crude antigen preparation disappears during formation of the precipitate Hence these findings preclude the antigen from being of a nucleic acid nature Furthermore, analysis of the precipitates by Dische's general cysteine sulfuric acid method did not give the positive pentose reaction shown for the antigen sample itself Thus, all of the analyses performed on this preparation suggest its antigenic nature to be primarily a property of the protein content This is further confirmed by the loss of precipitability with antiserum after tryptic digestion

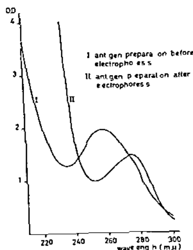



*Fig 5*

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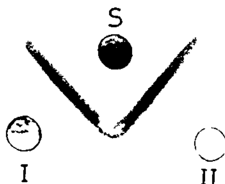



*Fig 6*
"Reaction of indentity" between the antigen sample purified by electrophoresis (I) *and the antigen A* prepared by Jensen *(II)* S = serum T L Wadsworth's technique

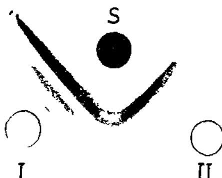



*Fig 7*
"Reaction of identity" between the protein fraction of Verwey (I) and the antigen A prepared by Jensen (II) S = serum T L Wadsworth's technique

gel As shown in Fig 7 this antigen fraction also gave "reaction of identity" with the sample received from Jensen Beside this finding it is obvious that both preparations contain more than one precipitable antigen, although this is most pronounced in the fraction obtained with Verwey's method

No chemical analysis of the Verwey antigen preparation has been performed in this study *Verwey* (1) has characterized the antigen fraction as being of a protein nature Thus, the substance gave a strongly positive *biuret reaction but a weak reaction for saccharides (Molisch* test) Furthermore, it contained about *14* per cent nitrogen *and less* than 0 1 per cent phosphorous Pentoses were not detectable The anti genic properties were destroyed by tryptic digestion These results are in agreement with ours for the electrophoretically purified antigen A-preparation

## DISCUSSION

*Jensen's* finding of antibodies in normal human sera against an antigen (antigen A) present in the majority (75 per cent) of *S aureus* strains is of considerable interest (3, 4) Jensen has suggested that the antigen is a polysaccharide The investigations in our laboratory do not confirm this A further purification of Jensen's antigen A-preparation has been carried out by electrophoresis Thereafter, analysis of the solution containing antigen A indicated the antigen being primarily composed of protein Jensen's observation that the antigen product gave a negative biuret reaction is difficult to explain, on the other hand the positive carbazole reaction he obtained was probably due to the pentoses of the nucleic acids present in his preparations Analysis of the preparation purified by electrophoresis did not show any nucleic acids or saccharides Thus pentoses or other carbohydrates could not be detected by Dische's general cysteine-sulfuric acid reaction, nor could purines and pyrimidines be found by chromatography of a hydrolysate The disappearance of the ultra violet absorption peak at 260 m$\mu$ points in the same direction Chemical examination of the antigen antibody precipitates confirmed these results All of the analyses performed here suggest the protein content is the essential factor for the antigenicity This suggestion is further supported by the disappearance of the precipitin reaction after tryptic digestion

Very little chemical work has been done on the protein antigens of *S aureus* One of the methods for preparation of protein antigens from these bacteria is that of *Verwey* (1) The finding that Jensen's antigen A is probably of a protein nature suggested further experiments to find out if any serological similarities existed between antigen A and a protein antigen fraction prepared by the Verwey method from a strain rich in antigen A In Verwey's method an extract of crushed bacteria is precipitated at pH 3 5 and the fraction is found in the supernatant By contrast Jensen's antigen A is precipitated when the extract of the boiled bacteria is adjusted to pH 3 0 Though the two methods differ completely in this respect, serologically identical antigens can be demonstrated in the final products Verwey's initial step is a milder one since boiling may denature existing protein antigens Actually, antigen preparations obtained from extracts of disintegrated frozen bacteria show a greater number of antigens by agar gel precipitation (Fig 7) As a final remark regarding the (partial) serological similarity between Verwey's protein fraction type specific for Julianelle's type A strains (1) and Jensen's antigen A preparation *Cowans* results (19) may be recalled Investigating haemolytic staphylococci he found that 83 per cent of the strains belonged to Julianelle's type A, a figure of about the same order as that of Jensen for the occurrence of antigen A in *S aureus* (4)

The results given in this report encouraged us to carry out further

experiments to purify the antigens of *S aureus* These results will be published elsewhere

## SUMMARY

The antigen A from *Staphylococcus aureus* described by *K Jensen* contains protein and is serologically identical to an antigen found in the protein fraction (fraction B) described by *Verwey*

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*FROM THE RICKETTSIA AND VIRUS DEPARTMENT STATENS SERUMINSTITUT, COPENHAGEN*

# STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

## *A Preliminary Report on Adoptive Immunization of Virus Carrier Mice*

*By*

MOGENS VOLKERT

Received 16 v 62

Except for certain tumor viruses, the only experimental evidence published hitherto which could indicate acquired immunological tolerance to a self replicating agent consists of reports concerning the virus of lymphocytic choriomeningitis (LCM) More than twenty years ago *Traub* (9) reported that if mice were infected with this virus intra utero or shortly after birth they developed a lifelong, symptomless, virus carrier state However, if mature mice were infected the infection would run a normal course, ending in either the death of the animal or recovery and clearance from the organs of any detectable virus Further reports from Traub and others (10, 11, 12, 2, 4, 13) have described in more detail the LCM virus infections initiated in both mature and premature mice The conclusion of all the reports seems to be that at least one important criterion for considering the non-responsive lifelong LCM virus carrier state to be due to tolerance is fulfilled, *viz* that the non-responsive LCM virus infection can be brought about by premature exposure of the animals to the virus However, a tolerance state is characterized not only by the fact that it can be brought about by premature exposure of the animal to an antigen, but that tolerance can also be terminated by adoptive immunization, i e by injecting the tolerant animal with spleen and lymph node cells from normal, or even better, immune animals Experiments concerning this second criterion for tolerance to the LCM virus have never been reported and therefore until more has been done to clarify this problem doubt still would prevail as to the role played by the tolerance phenomenon in the LCM virus infection

The question whether an immunological tolerance to a virus can be developed is of more than theoretical interest Firstly, if a tolerance state to one virus really exists, this would point to the possibility of tolerance to other viruses As it cannot be excluded that in certain virus

The author is indebted to *Dr F Traub* (Tübingen Germany) who has kindly provided the LCM virus strain and to *Miss B Højer* for skilled technical assistance

infections an excessive reaction of the host against the virus is the direct cause of death (*Rowe* (8)), development of a tolerant state to these viruses might perhaps be one of the protective mechanisms of the organism against the harmful effect of the infection (3) Secondly, if a virus carrier state developes because of the non-responsiveness of the tolerant animals, then by titrating the virus in the blood and the organs, a new possibility will exist for measuring the effect of certain procedures which influence the tolerance state Animals tolerant to a virus might therefore be valuable for immunological studies

For these reasons, the question of tolerance to viruses was taken up and the LCM virus carrier state in mice chosen as the first object of study It is the purpose of this article to describe the preliminary results of adoptive immunization experiments on LCM virus carrier mice

## MATERIAL AND METHODS

*The LCM virus strain* used throughout was provided by dr *Traub* In this laboratory the virus was passed twice in white mice of ordinary laboratory stock and then twice in AKA mice Subsequently the virus was passed exclusively in AKA mice when necessary

*The mice* used as LCM virus carriers and as donors were all from the same highly inbred strain of AKA mice These employed for virus titrations were all from the Institute s stock of ordinary white mice At the time of use their weight was between 12 and 14 grammes

*The virus titration* endpoints were calculated according to the method of Reed and Muench (7) and all titrations were carried out by intracerebral inoculations For practical purposes only whole logarithmic values are used in the figures Each of these was reached either by rounding up if the logarithmic decimal were higher than 5 or by rounding down if they were lower than 4

*Cell suspensions* for transplantation were mixtures of cells from the spleen and lymph nodes The organs were cut with scissors passed through a tissue sieve washed twice with and finally suspended in Hanks balanced salt solution containing two per cent lactalbumin one per cent glucose and tissue culture concentrations of penicillin and streptomycin Dependent on size each donor mouse gave between 100 and 200 million cells and of these roughly about two fifth were from the lymph nodes

## EXPERIMENTAL

### *Effect of Transplantation of Spleen and Lymph Node Cells from Immune Mothers on the LCM Virus Carrier State of their Offspring*

The virus carrier state in the mice was brought about as described by *Traub* (9) by infecting the new born AKA babies with the LCM virus In the present experiments the baby mice were inoculated within the first 18 hours after birth Each baby received a $10^5$ LD 50 dose of virus intraperitoneally in a volume of 0 05 ml In order to exclude any other antigens than the virus antigen viruses passed in AKA mice were used throughout Within the first 2–3 weeks after the virus inoculation, about 25 per cent of the babies died but the rest became virus carriers When used the mice were about three months old and were healthy looking but all had a virus titer in their blood $\geqq 10$

The mothers of the virus carrier babies were chosen as donors Traub

has shown that virus carrier babies excrete large amounts of virus and infect their mothers. Usually such infections would run a symptomless course and end in solid immunity. The present experiments seem to confirm this. No clinical symptoms of disease were seen among the mothers of the infected babies, but a group selected at random about three months after the babies were born showed complete immunity to a challenge of $10^3$ LD 50 doses of virus given intracerebrally. Therefore, the mothers of infected babies could be expected to be a good source for the supply of immune cells. In all cases the cells for transplantation were taken from mothers about three months after they had given birth and about six weeks after the babies were taken away from them. Thus all donors would have had a good chance of freeing themselves from any virus which might interfere with the transplantation results.

Sixteen virus carrier mice were selected at random before the experiment. The animals were bled from their tails and a check made on the virus titer in the blood of each mouse. As seen from Figure 1, A and B, all 16 mice had a virus titer in the blood $\geqq 10^2$.

The mice were then divided into groups with eight animals in each. The first group served as control and these mice received no treatment. Mice in the second group were injected with spleen and lymph node cells from the donors. All transplantations were carried out on the same day with cells from the same pool. The cell suspensions were given intravenously and all of the mice in this group received $100 \times 10^6$ cells in a total volume of 0.5 ml. As it was feared, as mentioned above, that the possible presence of virus in the transplanted cell suspension might interfere with the transplantation results, the cell pools used were tested for virus. However, no trace of virus was found.

Two weeks after the transplantation five mice in the control and five in the transplanted group were bled from their tails and the blood tested individually for virus. The findings are recorded in Figure 2, A and B, and show that whereas the titers for the control mice in all cases were, as before, $\geqq 10^2$, one of the transplanted group had a titer of $10^1$ and another a titer even less.

Four weeks after the transplantation all of the mice in both groups were bled from their tails and the blood tested individually for virus. As will be seen from the data given in Figure 3, A and B, the virus titers for the control mice were as before $\geqq 10^2$ in every case. However, in the transplanted group four out of the eight mice had titers lower than $10^1$.

Six weeks after the transplantation all of the mice in both groups were killed and full titration of the virus in both blood and spleen carried out for each mouse individually. The results are given in Figures 4 and 5, A and B. The data presented show that complete titration revealed a virus titer in the blood of $10^4$–$10^5$ for all of the animals in the control group, whereas those in the transplanted group had titers only of $10^2$ or less. As regards the virus content of the spleen, the

infections an excessive reaction of the host against the virus is the direct cause of death (*Rowe* (8)), development of a tolerant state to these viruses might perhaps be one of the protective mechanisms of the organism against the harmful effect of the infection (3). Secondly, if a virus carrier state developes because of the *non responsiveness of the tolerant animals*, then by titrating the virus in the blood and the organs a new possibility will exist for measuring the effect of certain procedures which influence the tolerance state. Animals tolerant to a virus might therefore be valuable *for immunological studies*.

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The mothers of the virus *carrier babies were chosen as donors*. Traub

difference between the control and the transplanted group was even more striking As regards the mice in the control group the spleen suspension showed in all cases virus titers $\geqq 10$ but as regards the mice in the group in which transplantation had been made none had titers higher than $10^{1}$ three out of the eight mice in this group had spleen titers below $10^{\circ}$

## CONCLUSION AND SUMMARY

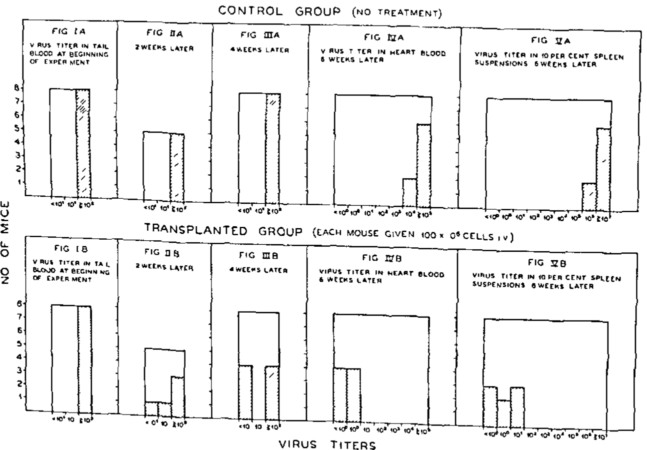
Acquired immunological tolerance (1 5 6) is not an all or nothing phenomenon but exists in every degree It cannot be distinguished clearly from other types of non responsiveness and interpretation of the phenomenon is still pure guesswork For these reasons it is not yet easy to prove that an observed immunological non responsiveness to *e g* a virus is caused by the immunological state designated tolerance

As mentioned in the introduction Traub and others have shown that one criterion is fulfilled for considering the non responsive lifelong LCM virus carrier state in mice to be due to the tolerance phenomenon The experiments described in this paper show that transplantation of immunological competent cells to LCM virus carrier AkA mice causes the virus titers in the blood and the spleen of the recipients to decrease In a few cases even a total clearance of the virus carriers of their virus seems to have been almost achieved

Even highly immune mice have never more than traces of antibodies to the LCM virus in the blood (3 8) Moreover the cells used in our experiment had been washed twice before they were transplanted For these reasons it seems unlikely that the effect on the virus could be caused by antibodies transferred together with the cells The slowly progressive decrease of the virus titer through six weeks points to an activity of living cells and in some ways our preliminary results are similar to what is seen when adoptive immunization is used to terminate a tolerance state to tissue transplants Thus the data presented here might indicate that also a second important criterion can be fulfilled for considering the LCM virus carrier state in mice to be due to tolerance and therefore they support the assumption that tolerance to the LCM virus does exist

In addition our observations indicate the possibility of effecting a cure of a virus carrier state in animals by experimental procedures

The type and numbers of cells used in the experiments described here had an effect on the virus but did not cause any clinical signs of disease However experiments in progress in this laboratory indicate already that adoptive immunization of virus carriers will not always run a symptomless course The grafting of genetic compatible lymfoid cells can also provoke a serious even fatal disease in the virus carriers with symptoms which might suggest a runtlike disease However until more data are available concerning this and the other problems further discussion is premature




*Figs 1–5*

The effect of adoptive immunization on virus carriers.

The big open squares indicate the numbers of mice titrated (along the ordinate) and the limits of the titrations (along the abcissa)

The black columns within the squares indicate the numbers of mice with the respective virus titers

*FROM THE VIRUS DEPARTMENT, NATIONAL INSTITUTE OF PUBLIC HEALTH, OSLO NORWAY (HEAD O LAHELLE, M D)*

# THE NORMAL SERUM COFACTOR IN THE HAEMAGGLUTINATION INHIBITION OF INFLUENZA $A_2$-VIRUS OF LOW ANTIBODY SENSITIVITY BY EARLY CONVALESCENT FERRET SERUM

*By*

ARILD HARBOE and ODD STRANDLI

Received 30 v 62

*Styk & al* (18) found that a heat labile, normal serum factor was necessary for the haemagglutination inhibition (HI) of influenza $A_2$-virus by serum from $A_2$-infected mice Later they reported (17) that the cofactor was necessary only for the inhibition of $A_2$-virus of low avidity Neither was the cofactor necessary for the inhibition by serum from hyperimmunized mice After these papers *Styk & al* published several others dealing with the cofactor discovered by them (3, 4, 11, 14, 15, 16)

*Harboe & Reenaas* (6) examined in the HI-test heated serum samples drawn from $A_2$-convalescent ferrets at different intervals after the infection They found that when the test virus was of a high avidity, or antibody sensitivity (a s ) as they suggested it might be called, the titre peak was recorded several days earlier than it would have been if a virus of a low a s was employed In a later investigation (5) a heat lability of the early ferret serum antibodies inhibiting virus of a low a s was found In the present investigation it has been examined whether the normal serum cofactor discovered by *Styk & al* is responsible for this lability and some properties of the cofactor have been studied

## MATERIALS AND METHODS

*Viruses* Influenza $A_2$ strains and an $A_1$ strain were employed references have been given previously (5 6) The viruses were grown in embryonated eggs

*Sera* The normal ferret or mouse sera were pooled from eight or more animals The sera were stored frozen

*Haemagglutination inhibition (HI) test* This was performed in plastic plates according to a standard method (9) The treatment of sera [illegible]

[illegible]

The technical assistance of Mrs *B Egelandsdal* and Miss *A Palmadottir* was appreciated

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as at 1 8 after the addition of the same volume of saline, while at 1 6 with the diluted normal ferret serum Similarly the titre of the antigen of high a s was 8 with saline only, and 4 when the diluted mouse serum was added

TABLE 1

*Haemagglutination Inhibition Test with Convalescent Sera from a Ferret Infected with $A_2$/Japan/305/57 EFMF*

| Days after inoculation | *Treatment of the convalescent sera* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | None | | | Heated at 56° C for one hour | | | Treated with cholera filtrate and heated at 56° C for one hour | | |
| Tested after addition of | *Saline* | Normal *ferret* serum | Normal *mouse* serum | Saline | Normal *ferret* serum | Normal mouse serum | Saline | Normal *ferret* serum | Normal mouse serum |
| 0 | < 12 | < 12 | < 12 | < 6 | < 12 | < 12 | < 6 (< 12) | < 12 | < 12 (< 12) |
| 7 | < 12 | < 12 | < 12 | < 6 | < 12 | < 12 | < 6 (288) | < 12 | < 12 (384) |
| 11 | 18 | 24 | 24 | < 6 | 24 | 12 | < 6 (768) | 24 | 12 (1152) |
| 16 | 48 | 48 | 48 | 18 | 48 | 48 | 18 (768) | 48 | 48 (1152) |
| 26 | 48 | 48 | 48 | 24 | 48 | 48 | 18 (384) | 48 | 48 (768) |

The sera had been subjected to different kinds of pretreatment and the effect of these treatments and of the addition of normal sera are shown Titres against $A_2$ virus of low antibody sensitivity are outside the brackets while titres against $A_2$ virus of high antibody sensitivity are inside

Table 1 shows that the addition of active, normal mouse serum is of practically no influence on the $A_2$-antibody titres recorded with the virus of a high a s (Neither was any influence seen in an analogous experiment with human convalescent sera performed in an attempt to increase the sensitivity of the diagnostic method)

With the virus of low a s, however, the results are seen to be entirely different Here the addition of active, normal sera has given a distinct titre increase of the pretreated sera The relative increase appears to be more pronounced in the 11 days than in the later sera – As might be expected, the addition of these diluted, active, normal sera has not exerted any distinct influence on the titres of the active, convalescent sera

It is further noted (Table 1) that in tests with virus of low a s on active sera, the course of the antibody development recorded is *more* similar, though still not identical, to the course recorded with virus of a high a s, than is the course recorded with pretreated sera in the absence of active, normal sera This tendency was regularly found in the seven animals studied, and agrees with the results presented in Table 1 of a previous paper (5)

The experiments confirm the finding made by *Styk & al* (17, 18) that there is a cofactor in normal, unheated serum which can restore

*Receptor destroying enzyme (RDE)* This was prepared from cholera filtrate (Philips Duphar) by one cycle of absorption to fowl red cells and subsequent elution into calcium acetate saline buffer, pH 6 2 (2 8) The titre of the semi purified RDE preparation was 10 times the titre of the original crude cholera filtrate, the test virus was PR-8 (2, 8)

*Trypsin* Trypure "Novo" was employed It is a crystalline preparation which contains 25 Anson units per g Immediately before use the enzyme was dissolved in M/15 phosphate buffer, pH 8 0

## RESULTS

### 1 *Reactivation of $A_2$-Antibodies in Heated, Convalescent Serum by Addition of Active, Normal Serum*

In these experiments the HI-test was performed with sera from seven ferrets which were convalescent from an $A_2$-infection The sera were subjected either to heating at 56° C for one hour, or to treatment with cholera filtrate according to the standard method (19), this involved a final heating of the enzyme-treated at 56° C for one hour The sera were drawn at different intervals after the infection with $A_2$ virus, which was performed by intrasanal instillation of infected egg allantoic fluid The strains were $A_2$/Japan/305/57 and $A_2$/Singapore/1/57 The sera were examined in the absence and presence of active, normal ferret or mouse serum Test antigens were an egg line (E) and an egg-ferret-mouse-egg line (EFME) of $A_2$/Japan/305/57, the former line being of low, the latter of high antibody and normal-inhibitor sensitivity (6, 10) The HI-test was performed as follows To 0 25 ml of a serial 2-fold dilution of the convalescent sera (active or pretreated) was added 0 25 ml of active, normal ferret or mouse serum diluted 1 20, or of saline, in all 3 parallel tests It was necessary to dilute the active, normal sera in order to make their haemolytic activity less disturbing in the HI-test This could also be achieved by performing the test at +4° C instead of at room temperature At the low temperature, however, the haemagglutination inhibition was reduced so much that the virus of low antibody sensitivity regularly failed to record any antibody activity in the cholera treated sera, and it was therefore decided to perform these experiments at room temperature (about 18° C) About ½ hour after the active, normal sera had been added, 0 25 ml of 0 5 per cent red cells and immediately afterwards 0 25 ml of the virus suspension were added – Normal inhibitor activity made it impossible to examine ferret sera with the virus of high a s , unless they had been treated with cholera filtrate Normal mouse serum, however, is practically devoid of $A_2$-inhibitors (8, 12) and could therefore be tested on cofactor activity

The titres presented in Table 1 are representative of the results obtained with the seven ferrets The titres recorded in the table are the primary serum dilutions which give partial haemagglutination inhibition Of the virus of low a s a test antigen was employed which gave partial haemagglutination at the dilution 1 8 after addition of 0 25 ml of the diluted mouse serum to the titration of agglutinating doses, as well

sistant to an RDF treatment which heavily reduces the $\alpha$ inhibitor activity in the serum

TABLE 2
*The Effect of Treatment with Receptor Destroying Enzyme (RDE) on the Cofactor and the Normal Inhibitors in Ferret Serum*

| Treatment of the normal serum | Cofactor titre | Normal inhibitor titre against | | |
|---|---|---|---|---|
| | | $A_2$ Japan 305 57 EFML | Heated Lee | $A_1$ Persian Gulf 2 52 P-line |
| Incubation over night at 37° C pH 6 2 with RDE | 384 | 36 | 36 | 576 |
| Incubation with the buffer only | 384 | 384 | 1536 | 768 |
| None | 384 | | | |
| Heating at 56° C for one hour | <6 | | | |

3 *The Effect of Treatment with Trypsin on the Cofactor and on the $\alpha$ Inhibitor Activity*

The treatment with trypsin was performed by adding 4 volumes of different dilutions of trypsin in buffer to 1 volume of active, normal ferret serum and keeping the mixtures at 37° C over night After this treatment the routine absorption with 10 per cent fowl red cells at 0° C was performed, except in the aliquots to be tested on haemagglutinating lipids, where absorption was not performed The determination of the cofactor activity was performed in essentially the same way as in the experiments above The titration of haemagglutinating serum lipids released by the trypsin treatment (7) was performed by adding 0 25 ml of a 0 5 per cent suspension of vaccinia positive fowl red cells (from a White Leghorn rooster) to 0 25 ml of a 2 fold dilution series of each of the trypsinized serum preparations The results are presented in Table 3

4 *The Effect of Absorption with an $A_1$-Strain on the Cofactor and on the $\beta$ Inhibitor Activity*

A pure egg line of the strain $A_1$ Persian Gulf/2 52 in the P phase was employed The virus material used for absorption was prepared by high speed centrifugation (25 000 G) of infected chick allantoic fluid Normal mouse serum was treated with about 10 times the amount of virus material necessary to remove the $\beta$ inhibitor The serum-virus mixture was kept for one hour at room temperature, and subsequently spun for 2 hours at 25,000 G at +4° C One volume of packed, fowl red cells was added to 9 volumes of the supernatant, and the suspension kept for 30 minutes at 0° C before the cells were spun down After this treatment, which was the routine method for the removal of haemagglutinins, the serum was no longer haemagglutinating

the antibody activity against $A_2$-virus of low a s, when this activity has been reduced by heating – In the following experiments the chemical nature of the cofactor was studied

*2 The Effect of Treatment with RDE on the Cofactor and on the α-Inhibitor Activity*

One volume of normal ferret serum was incubated over night at 37° C with 5 volumes of semipurified RDE Subsequently the enzyme was removed from the serum by absorbing 3 times with fowl red cells The absorption was performed by adding 1 volume of packed cells to 9 volumes of serum, 3 minutes later the cells were spun down and discarded These procedures took place at 0°–4° C It was checked that the absorbed, RDE-treated sera failed to enhance the elution of virus from the red cells

The HI-tests in the following experiments were performed at +4° C in order to reduce haemolysis by the unheated sera In an initial test the optimal dilution of the indicator serum (14) was determined This serum consisted of a pool of equal volumes of serum samples drawn from 35 ferrets 12 days after intrasanal instillation of one ml of $A_2$/Japan/305/57 EFME infected, chick allantoic fluid The test antigen was $A_2$/Japan/305/57 E, 6 haemagglutinating doses were employed It was then found that the active indicator serum gave the titration endpoint (*i e* partial inhibition) at the primary serum dilution 1 72, while after heating at 56° C for one hour the endpoint was found at 1 12 When the heated indicator serum was tested in the presense of active, normal ferret serum diluted 1 20, the endpoint was at 1 96 This showed that the heating had reduced the cofactor activity only, the specific $A_2$-antibody activity had not been impaired It was then decided to employ the heated indicator serum at the dilution 1 30 in the following titrations of cofactor activity

In these titrations were compared Untreated and RDE-treated serum and also a control preparation consisting of the normal serum diluted 1 6 with the calcium acetate saline buffer and incubated over night at 37° C In quantities of 0 25 ml the serial 2-fold dilutions of the three preparations were put up in the plastic plates, subsequently 0 25 ml of the diluted, heated indicator serum was added to each groove, and finally the same suspension of red cells and of test virus as above, also in 0 25 ml amounts The results are shown in Table 2 As regards the content of cofactor no difference is seen between the RDE-treated serum, the buffer treated, and the untreated sera On the other hand, as regards the normal inhibitor activity there is a pronounced difference between the former one and the latter two That RDE, and not tryptic enzymes, had been active in the enzyme preparation was checked in a test against a β-inhibitor sensitive $A_1$-strain, which recorded no distinct difference The experiments therefore demonstrate that the cofactor is re-

The cofactor appears to be entirely different from the $\alpha$ inhibitor in serum The former is heat labile and resistant to RDE, the latter is heat stable and sensitive to RDE (Tables 2 and 3) The influence of RDE on the cofactor was not examined by *Styk & Hana* (15)

The cofactor was found to be destroyed by trypsin It is seen in Table 3, that when the trypsin concentration of the mixture is 0 64 or 0 32 per cent, no cofactor activity is left, the $\alpha$-inhibitor activity is considerably reduced, and the haemagglutinating lipids are active At 0 16 per cent, however, there is only a minor reduction of cofactor and $\alpha$ inhibitor activity, and the haemagglutinating lipids are still neutralized

The failure of the trypsin to destroy the cofactor in the experiments of *Styk & Hana* (15) is puzzling Perhaps this failure is due to the trypsin inhibitors in the sera, these inhibitors may have destroyed a low potency of the trypsin preparation employed *Styk & Hana* further report (15) that when trypsinized sera are tested, it is not possible to use the erythrocytes from any rooster, as cells from some of these are agglutinated by trypsin even in high dilutions Their observation may be explained by the finding reported in a previous (7) and the present paper, that the cells which were agglutinated to high dilutions by trypsinized sera, were obtained from vaccinia positive fowl This finding suggested that the haemagglutination shown by trypsinized sera is caused by normal serum lipids, whose anti haemagglutinating proteins have been digested by the enzyme In unpublished experiments the authors found that this haemagglutination can be destroyed when untreated serum is added

*Hana & Styk* found (4) that cofactor can be separated from $\beta$ inhibitor by chromatography on DEAE cellulose That the cofactor is different from the $\beta$ inhibitor was confirmed in the present investigation It was found that the $\beta$-inhibitor could be removed from serum by absorption with $A_1$ virus, while the cofactor remained


## SUMMARY

The heat labile, normal serum cofactor necessary for the haemagglutination inhibition of influenza $A_2$ virus of low antibody sensitivity by early convalescent ferret serum was studied

The absence of cofactor was found to be partly responsible for the decrease in the ratio between titres to $A_2$-virus of high and of low antibody sensitivity observed in heated ferret sera during the course of the convalescence

The cofactor was resistant to RDE, and sensitive to trypsin When the $\beta$ inhibitor was removed from the serum by absorption with $A_1$-virus the cofactor remained

The titre of the cofactor before and after the serum had been treated with the virus, was determined as above by means of early $A_2$-convalescent ferret serum and $A_2$/Japan/305/57 E. The titres of the β-inhibitor were tested with the $A_1$-strain. The results are shown in Table 4.

TABLE 3

*The Effect of Treatment with Trypsin and of Heating on the Cofactor and the α-Inhibitor in Ferret Serum. The Release of Haemagglutinating Serum Lipids by the Trypsin Treatment is also Shown*

| Treatment of the normal serum | Cofactor titre | Normal Inhibitor $A_2$ Japan 305/57 I.E.M.H. | Titre against Heated Lee | Titre against vaccinia positive fowl red cells |
|---|---|---|---|---|
| *Incubation over night at 37° C,* | | | | |
| pH 8.0 with 0.64 % trypsin | < 10 | 40 | 80 | 4800 |
| with 0.32 % trypsin | < 10 | 40 | 80 | 6400 |
| with 0.16 % trypsin | 40 | 120 | 640 | < 100 |
| with 0.08 % trypsin | 60 | 120 | 640 | < 100 |
| with 0.04 % trypsin | 60 | 160 | 640 | < 100 |
| with 0.02 % trypsin | 80 | 160 | 640 | < 100 |
| with the buffer only | 80 | 160 | 960 | < 100 |
| None | 80 | 160 | 640 | |
| Heating at 56° C for one hour | < 10 | 640 | 1920 | |

TABLE 4

*The Effect of Absorption with an $A_1$-Strain on the Cofactor and the β-Inhibitor in Mouse Serum*

| Treatment of the normal serum | Cofactor titre | Normal inhibitor titre against $A_1$ Persian Gulf 2 2 P line |
|---|---|---|
| *Absorption with $A_1$/Persian Gulf/2 52 P line* | 60 | < 5 |
| None | 80 | 60 |
| Heating at 56° C for one hour | < 5 | < 5 |

## DISCUSSION

The results of the present investigation confirm the observation by *Styk & al* (17, 18) of the existence of a heat labile, normal serum co factor active in HI-tests with $A_2$-virus of a low a s. It was found (Table 1) that destruction of the cofactor is partly responsible for the decreasing ratio between titres recorded with virus of high and virus of low a s, when serum samples drawn during the course of the convalescence are tested after heating, and in particular *after treatment with* cholera filtrate followed by heating (5, 6). Also in the active sera this ratio was *found* to decrease, though here less markedly. Whether the decrease might be eliminated if a great excess of cofactor was added, could not be tested because of haemolysis.

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# INVESTIGATION INTO THE BACTERIOSTATIC AND FUNGISTATIC EFFECTS OF CERTAIN ANTIMYCOTIC SUBSTANCES IN AQUEOUS SOLUTIONS AND INCORPORATED IN OINTMENTS

*By*

OLE G CLAUSEN

Received 7 v 62

The following is an account of a comparative investigation into the bacteriostatic and fungistatic effects – in aqueous solutions and in a polyethyleneglycol ointment base – of salicylanilide (*Clausen* 1962), cetylpyridiniumchloride, and a combination of these two substances in comparison with the effects of a number of known and currently employed substances and preparations The investigations include, among other things, a study on the effects of the substances when the pH value is varied in the ointment preparations

The reference substances and reference preparations employed were the following undecylenic acid, Loramine Du 185 (dialkylolamides of undecylenic acid, manufactured by Dutton & Reinisch Ltd, London), Thioxolon = 4 hydroxy-1 3-(2H) benzoxathiol-2-on (*Kjeldgaard* 1960), Amphotericin B (Fungizone "Squibb"), Griseofulvin, I C I, Decylene cream (made up at a chemist's), and Mycostatin ointment, AB Bofors ("Squibb' Licence)

## MEDIA AND METHODS

The test microbes employed are listed in Table 1, page 322 In addition to these test organisms, for ointment No 7 (and to some extent for ointment 8), Table 3, page 324, the following fungus strains were also utilized Cryptococcus neoformans, Geotrichum candidum, Aspergillus niger, and yet another strain of Trichophyton mentagrophytes

Thus the test microbes used in the investigation comprise pathogenic bacteria and species of fungus which are of the most chemo-resistant kinds, while several of them are highly important microbes in cases of skin infection

The investigations were carried out with the aid of a diffusion

I should like to express my sincere thanks to my two assistants *Mrs Kari Raugstad* and *Mrs Eva Lonstad* both of whom have performed excellent work in helping to carry out the experiments

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Received 7.v.62

The following is an account of a comparative investigation into the bacteriostatic and fungistatic effects – in aqueous solutions and in a polyethyleneglycol ointment base of salicylanilide (*Clausen* 1962), cetylpyridiniumchloride, and a combination of these two substances in comparison with the effects of a number of known and currently employed substances and preparations. The investigations include, among other things, a study on the effects of the substances when the pH value is varied in the ointment preparations.

The reference substances and reference preparations employed were the following: undecylenic acid, Loramine Du 185 (dialkylolamides of undecylenic acid, manufactured by Dutton & Reinisch Ltd, London), Thioxolon = 4 hydroxy-1,3-(2H)-benzoxathiol 2 on (*Kjeldgaard* 1960), Amphotericin B (Fungizone "Squibb"), Griseofulvin, I.C.I., Decylene cream (made up at a chemist's), and Mycostatin ointment, AB Bofors ("Squibb" Licence).

## MEDIA AND METHODS

The test microbes employed are listed in Table 1, page 322. In addition to these test organisms, for ointment No. 7 (and to some extent for ointment 8), Table 3, page 324, the following fungus strains were also utilized: Cryptococcus neoformans, Geotrichum candidum, Aspergillus niger, and yet another strain of Trichophyton mentagrophytes.

Thus the test microbes used in the investigation comprise pathogenic bacteria and species of fungus which are of the most chemo-resistant kinds, while several of them are highly important microbes in cases of skin infection.

The investigations were carried out with the aid of a diffusion

method, the solutions and ointments being *introduced into holes (1 0 cm in diameter)* made in blood agar media, which had in advance been inoculated on the surface with specific diluted cultures of the particular test microbe concerned, and then dried

The bacterium inocula were obtained from 20-hour cultures (37° C) in ordinary beef-infusion peptone broth by diluting in sterile distilled water in the following proportions, expressed in ml inoculum per ml *dilution*: *Ps aerug* 1 : 500, *E coli* 1 : 300, Str faec 1 200, S aureus 1 200

The fungus inocula *were obtained by suspending 10-day-old cultures*, cultivated on Sabouraud agar at 25° C, in the broth referred to above, in mortars The Candida, Geotrichum, and Cryptococcus inocula contained *c* 50 million living cells per ml, and those of the Aspergillus strains *c* 10-20 million living spores per ml The inocula were diluted with sterile distilled water in the following proportions C albicans 1 40, Crypt neoform and Geotrich cand 1 20, Asp flav and Asp fumigat 1 12 5

A 10-day-old Sabouraud agar slant culture of Tr mentagrophytes was suspended *by mortar in 20 ml beef-infusion peptone broth This* suspension was used undiluted as inoculum

For the bacterium tests recourse was had to 10 per cent peptone-free horse blood agar with a pH value of 7 4 (Japanese agar, quality Kobe I) The media were incubated for 20 hours at 37° C, whereupon the diameters of the inhibition zones were registered

For the purpose of the fungus tests 1 per cent glucose was added to the afore-mentioned medium The cultures of C albicans and Asp flavus were incubated for 20 hours at 37° C The cultures employing Asp *fumigatus as a test strain were incubated for 30 hours at 37° C, while* Geotrich cand was incubated for 40 hours at the same temperature The cultures with Crypt neoform and the two Tr mentogrophytes strains were incubated for two days at 25° C, before the diameters of the inhibition zones were registered Large petri dishes, some 13 5 cm in diameter, were used The media were *c* 0 7 cm in depth

It proved to be important that the holes made in the agar were carefully filled and *almost* up to the brim with ointment, *the presence of* even the minutest quantity of ointment outside the holes resulted in *uneven inhibition zones The technical performance of the tests demands* practice In the case of relatively solid ointments gentle heating sometimes proved necessary after transference of the ointment from the tube to sterile containers, and thence to the media by means of slightly warmed sterile glass tubes fitted with rubber teats

Salicylanilide and Thioxolon, and to a certain extent also *undecylenic* acid, are heavily soluble in water and were accordingly dissolved by adding NaOH N/1, and pH values in the strongest solutions, $10^{-2}$, were adjusted to 9 5–9 9, when necessary by means of HCl N/1 The dilutions $10^{-3}$ and $10^{-4}$ were prepared from the $10^{-2}$ dilutions, the pH value falling

somewhat as a result Dilution of the clear solution of salicylanilide, $10^{-2}$, pH 9 75, resulted in a lower pH and precipitation occurred in the dilutions $10^{-3}$ and $10^{-4}$ The pH values of these solutions were therefore readjusted to 9 10, and the salicylanilide then once again dissolved

Buffer solutions containing no antimicrobial substance, with a pH value between 5 and 10, were investigated parallel with the other test samples (see Addendum, page 322)

Of the ointment preparations listed in Table 3, page 324, Nos 1 to 6 were made by *Weider s Farmasoytiske A/S*, Oslo 7 and 8 by the author of this paper, 9 was made up at a chemist s, and 10 is a factory made preparation

The results of the investigations are set out in Tables 1, 2, and 3

*The Compositions of the Ointments Investigated*

*Ointments 1 2 and 3*

| 1 | | | 2 | 3 |
|---|---|---|---|---|
| | Salicylanilide | 5 0 g | Lacks cetyl pyridinium chloride otherwise as 1 | Lacks salicylanilide, otherwise as 1 |
| | Cetylpyridiniumchloride | 0 2 g | | |
| | Distilled water | 7 6 g | | |
| | Polyethyleneglycols up to | 100 0 g | | |
| | pH 4 3 | | pH 6 1 | pH 4 4 |

*Ointments 4 5 and 6*

| 4 | | | 5 | 6 |
|---|---|---|---|---|
| | Salicylanilide | 2 5 g | Lacks cetyl pyridinium chloride other wise as 4 | Lacks salicylanilide otherwise as 4 |
| | NaOH N/1 q s | 10 0 g | | |
| | Cetylpyridiniumchloride | 0 2 g | | |
| | Distilled water | 7 6 g | | |
| | Polyethyleneglycols up to | 100 0 g | | |
| | pH 8 8 | | pH 8 8 | pH 11 4 |

In addition to preparations 4 and 6 above the corresponding ointments containing 0 1 per cent cetylpyridiniumchloride were investigated

*Ointments 7 and 8*

| 7 | | | 8 | |
|---|---|---|---|---|
| | Salicylanilide | 2 5 g | Salicylanilide | 2 5 g |
| | NaOH N/1 q s | 13 5 g | Distilled water | 17 5 g |
| | HCl N 1 q s | | Polyethyleneglycols up to | 100 0 g |
| | (pH 10 6 10 7) | | | |
| | Distilled water | 4 0 g | | |
| | Polyetheleneglycols up to | 100 0 g | | |
| | pH 9 5 | | pH 3 8 | |

In the ointment samples the pH values were potentiometrically determined by dissolving 10 per cent ointment in sterile distilled water

*Ointments 9 and 10*

9 *Decylene cream* (N 4 F)

| | |
|---|---|
| Zinc undecylenate pulverized | 20 0 g |
| Undecylenic acid | 5 0 g |
| Polyethyleneglycols (Ungv macrogoli) | 70 0 g |
| Distilled water | 5 0 g |

10 *Mycostatin ointment AB Bofors* ( *Squibb Licence*)

Nystatin "Squibb 100 000 U per g

Polyethyleneglycols and paraffinum liqv q s

TABLE 1

*Determination of the Bacteriostatic and Fungistatic Effects of Salicylanilide and Certain other Antimycotic Substances*

| Test microbes | Salicylanilide | | | Undecylenic acid [1 2] | | | Thioxolon (4 Hydroxy 1 3 (2H benzoxathiol 2 on | | |
|---|---|---|---|---|---|---|---|---|---|
| | $10^{-2}$ pH 9 75 | $10^{-3}$ pH 9 1 | $10^{-4}$ pH 9 75 | $10^{-2}$ pH 9 75 | $10^{-3}$ pH 7 5 | $10^{-4}$ pH 6 6 | $10^{-2}$ pH 9 9 | $10^{-3}$ pH 8 0 | 10- pH 6 4 |
| Ps aerug | 1 75 (1 9) | 1 05 (+) | 0 | 0 (1 65)* | 0 (+)* | 0 (+) | 1 5 | 0 | 0 |
| E coli | 2 0 (+) | 1 05 (+) | 0 (+) | 0 | 0 | 0 | 1 5 | 0 | 0 |
| Str faecal | 0 (2 1) | 0 | 0 | 0 | 0 | 0 | 1 4 | 0 | 0 |
| S aureus, phage type 42 D | 3 2 (3 6) | 2 25 | 0 | 1 25 (1 9) | 0 | 0 | 1 4 | 0 (1 3) | 0 |
| C albicans | 3 0 (4 0) | 1 6 (2 5) | 0 (1 3) | 2 3 (2 8)§ | 0 (+) | 0 | 1 9 (2 5) | 0 (+) | 0 |
| Asp flav | 3 15 (4 9) | 1 7 (2 5) | 0 (+) | 2 2 (2 4) | 0 (1 3) | 0 | 1 9 (2 1) | 0 (+) | 0 |
| Asp fumigat | 3 9 (4 9) | 2 0 (3 25) | + (1 9) | 3 2 | 0 (1 7) | 0 | 2 3 (3 0) | 0 (2 0) | 0 |
| Tr mentagrophyt | 4 9 | 2 6 | 0 (+) | 3 7 | 0 (1 9) | 0 (+) | 2 9 | 0 (1 7) | 0 |

Cf the method described above

Absolute inhibition zones = zones totally devoid of growth

Relative inhibition zones = zones in which growth is distinctly inhibited to zones *which are almost devoid of growth*

The figures not in brackets relate to the diameters of *absolute* inhibition zones measured in cm, the figures in brackets relate to the diameters of *absolute + relative* inhibition zones measured in cm

+ represents small barely registrable absolute inhibition zones

(+) represents small barely registrable relative inhibition zones

0 designates no absolute inhibition zones

*The holes made in the media were 1 0 cm in diameter*

[1] Loramine Du 185 (dialkylolamides of undecylenic acid manufactured by Dutton & Reinisch Ltd London) was investigated parallel with undecylenic acid but was found to have a distinctly weaker effect than the latter

[2] Undecylenic acid was also investigated in dilutions 10 2 pH 7 5, 10 3 pH 6 5 10 4 pH 5 7 The results were on a par with those tabulated

* Very faint trace of fair sized relative inhibition zones c 4 2 3 2 cm in diameter

§ Very faint relative inhibition zone 3 8 cm in diameter *in addition to that entered in the table*

## ADDENDUM

*Griseofulvin* I C I was also investigated in regard to its effect on all the test microbes employed This antibiotic affected only the two Tr mentagrophytes test strains In the highest dilution used, $10^{-4}$, a saturated aqueous *solution with a little sediment, it caused only relative* inhibition zones

The effect was demonstrable up to $10^{-6}$

The buffer solutions, which contained no antimicrobial substance and which had a pH value of 5–10, caused no inhibition zones on the blood agar media with the test microbes employed

TABLF 2

*Comparative Investigation into the Fungistatic Fffects of Salicylanilide and Amphotericin B*

| Test microbes | Salicylanilide* | | | Amphotericin B (= Fungizone Squibb ) | | |
|---|---|---|---|---|---|---|
| | 1 2 $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | 1 2 $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
| C albicans | 2 8 (3 8) | 1 6 (2 5) | 0 (1 3) | 2 15 | 2 15 | 2 15 |
| Asp fumigat | 3 4 (4 5) | 2 0 (3 25) | + (1 9) | 2 6 | 2 2 | 2 0 |
| Tr mentagrophyt | 4 3 | 2 6 | 0 (+) | 1 5 | 1 7 | 1 7 |

Cf the text of Table 1

pH Values of the stock solutions Salicylanilide (½ 10 ²) pH 9 8 Amphotericin B (½ 10 ²) pH 7 4

* *Salicylanilide* had the following effects in two earlier non tabulated dilutions

| Test microbes | 1 4 $10^{-2}$ pH 9 0 | 1 8 $10^{-2}$ pH 8 7 |
|---|---|---|
| C. albicans | 2 2 (2 7) | 1 8 (2 5) |
| Asp fumigat | 2 9 (3 7) | 2 6 (3 5) |
| Tr mentagrophyt | 3 7 | 3 2 |

## DISCUSSION

From Table 1 page 322, it is evident that salicylanilide is by far superior to the other substances investigated with respect to antimicrobial effect This superiority is particularly striking in relation to the pathogenic fungus strains

From Tabl [illegible] the dilution ½ 10 [illegible] B (Fungizone Squil [illegible] salicylanilide was generally about as effective as Amphotericin B In the dilution $10^{-4}$ however Amphotericin B continued to be almost as effective as in the dilution ½ 10 ², whereas salicylanilide proved itself much less effective in the dilution $10^{-4}$ Thus salicylanilide has a high dilution coefficient whereas that of Amphotericin B is very low (The latter compound also showed itself to be effective even in the dilution $10^{-6}$)

Griseofulvin I C I was found to have a strongly selective effect, and in consequence must be unfitted for use as a constituent of a general antimycotic ointment

From the foregoing it seems clear that salicylanilide has a stronger effect by far than any of the other substances investigated in concentrations usable for external application

In Table 3 page 324 the effects of ten ointments are recorded Two of these Nos 9 and 10 are to be regarded as reference preparations they

TABLE 1

*Determination of the Bacteriostatic and Fungistatic Effects of Salicylanilide and Certain other Antimycotic Substances*

| Test microbes | Salicylanilide | | | Udecylenic acid [1,*] | | | Thioxolon (4 Hydroxy 1 3 (2H)-benzoxathiol 2 on) | | |
|---|---|---|---|---|---|---|---|---|---|
| | $10^{-2}$ pH 9 75 | $10^{-3}$ pH 9 1 | $10^{-4}$ pH 9 75 | $10^{-2}$ pH 9 75 | $10^{-3}$ pH 7 5 | $10^{-4}$ pH 6 6 | $10^{-2}$ pH 9 9 | $10^{-3}$ pH 8 0 | $10^{-4}$ pH 6 4 |
| Ps aerug | 1 75 (1 9) | 1 05 (+) | 0 | 0 (1 65)* | 0 (+)* | 0 (+) | 1 5 | 0 | 0 |
| E coli | 2 0 (+) | 1 05 (+) | 0 (+) | 0 | 0 | 0 | 1 5 | 0 | 0 |
| *Str faecal* | *0* (2 1) | *0* | *0* | *0* | *0* | *0* | *1 4* | *0* | *0* |
| S aureus phage type 42 D | 3 2 (3 6) | 2 25 | 0 | 1 25 (1 9) | 0 | 0 | 1 4 | 0 (1 3) | 0 |
| C albicans | 3 0 (4 0) | 1 6 (2 5) | 0 (1 3) | 2 3 (2 8)§ | 0 (+) | 0 | 1 9 (2 5) | 0 (+) | 0 |
| Asp flav | 3 15 (4 9) | 1 7 (2 5) | 0 (+) | 2 2 (2 4) | 0 (1 3) | 0 | 1 9 (2 1) | 0 (+) | 0 |
| Asp fumigat | 3 9 (4 9) | 2 0 (3 25) | + (1 9) | 3 2 | 0 (1 7) | 0 | 2 3 (3 0) | 0 (2 0) | 0 |
| Tr mentagrophyt | 4 9 | 2 6 | 0 (+) | 3 7 | 0 (1 9) | 0 (+) | 2 9 | 0 (1 7) | 0 |

Cf the method described above

Absolute inhibition zones = zones totally devoid of growth

*Relative inhibition zones = zones in which growth is distinctly inhibited to zones which are almost devoid of growth*

The figures not in brackets relate to the diameters of *absolute* inhibition zones measured in cm, the figures in brackets relate to the diameters of *absolute + relative* inhibition zones measured in cm

\+ represents small barely registrable absolute inhibition zones

(+) represents small barely registrable relative inhibition zones

0 designates no absolute inhibition zones

The holes made in the media were 1 0 cm in diameter

[1] Loramine Du 185 ( acid manufactured by Dutton & Reinisch Ltd I with undecylenic acid but was found to have a latter

[2] Undecylenic acid was [2] pH 7 5 $10^{-3}$ pH 6 5 $10^{-4}$ pH 5 7 The results were on a par with those tabulated

* *Very faint trace of fair sized relative inhibition zones c 4 2 3 2* cm in diameter

§ Very faint relative inhibition zone 3 8 cm in diameter in addition to that entered in the table

## ADDENDUM

*Griseofulvin* I C I was also investigated in regard to its effect on all the test microbes employed This antibiotic affected only the two Tr mentagrophytes test strains In the highest dilution used, $10^{-4}$, a saturated aqueous solution with a little sediment, it caused only *relative* inhibition zones

*The effect was demonstrable up to* $10^{-6}$

The buffer solutions, which contained no antimicrobial substance and which had a pH value of 5–10, caused no inhibition zones on the blood agar media with the test microbes employed

comprise one medicinal antimycotic cream and one ointment containing an antibiotic substance

The results show that the pH value of the ointment preparations is decisive in determining their effect This is because salicylanilide is soluble in water only as an alkali salt The best effect was achieved with ointment 7 containing salicylanilide 2½ per cent and with a pH of 9 5 but ointments 4 and 5 with the same salicylanilide content and a pH of 8 8 come close up to No 7 The addition of cetylpyridiniumchloride 0 1 per cent and 0 2 per cent does not appear to have any appreciable influence on the effect of the salicylanilide ointments within the pH range 8 2[1] to 8 8 It is possible however that the effect on streptococci increases somewhat in the presence of cetylpyridiniumchloride this is most clearly apparent in the case of the ointment with a pH value of 4 3 (No 1 in Table 3)

On the whole cetylpyridiniumchloride in the absence of salicylanilide proved rather more effective in a concentration of 0 2 per cent than in one of 0 1 per cent in the polyethyleneglycol ointment base

Several ointment preparations containing salicylanilide 2½ per cent and with or without cetylpyridiniumchloride were investigated with varying pH values[1] Generally speaking it may be said that these ointments vary little in their effects within the pH range 8 5 to 9 5 At a pH of 8 2 the effect is likewise very good and only a slight reduction in effect was registrable The ointments distinguished themselves by the particular excellence of their *fungistatic* effects

It must be stressed that a 2½ per cent salicylanilide ointment with a pH value of c 9 is considerably more effective than a 5 per cent ointment with a pH value of c 6 This is easily understandable from what has been said above On the other hand it is difficult to explain without making closer investigation why a 2½ per cent salicylanilide ointment with a pH of c 4 also has a better bacteriostatic and fungistatic effect than the 5 per cent ointment referred to above

The fact that the salicylanilide ointments (pH 8 8 to 9 5) have a far stronger effect than Decylene cream and the Mycostatin ointment is most remarkable It is realised that the the composition consistency and solubility in water of the ointment base are of great importance to usefulness of the method in investigating the antimicrobial effects of ointments and creams Attention is drawn also to the uniformly good effect of salicylanilide both in aqueous solutions and in the ointment base employed

It may be observed that some of the ointment preparations investigated (salicylanilide 2½ per cent pH originally 9 2 9 5) had been stored in tubes for one year at c 22° C without it being possible to prove with certainty that the antimicrobial effects of the ointments thereby suffered a reduction

[1] Not all these results have been tabulated

Table 3

*The Bacteriostatic and Fungistatic Effects of Nine Ointments and One Cream Preparation*

| Test microbes | 1<br>Sal 5 %<br>Cet 0 2 %<br>pH 13 | 2<br>Sal 5 %<br>pH 6 1 | 3<br>Cet 0 2 %<br>pH 4 4 | 4[a)]<br>Sal 2 5 %<br>Cet 0 2 %<br>pH 8 8 | 5<br>Sal 2 5 %<br>pH 8 8 | 6[b)]<br>Cet 0 2 %<br>pH 11 4 | 7[c)]<br>Sal 2 5 %<br>C<br>pH 9 5 | 8[d)]<br>Sal 2 5 %<br>C<br>pH 3 8 | 9<br>Decylene cream | 10<br>Mycostatin ointment Bofors |
|---|---|---|---|---|---|---|---|---|---|---|
| Ps aerug | 0<br>(1 5) | 0<br>(1 6) | 1 05 | 0<br>(2 4) | 0<br>(2 4) | 1 2 | 1 9<br>(2 4) | 0<br>(1 6) | 0<br>(1 5) | 0 |
| E coli | 1 2<br>(1 5) | 1 15<br>(1 4) | 1 3 | 1 7<br>(2 0) | 1 7<br>(2 0) | 1 3 | 2 2 | 1 25 | +<br>(1 3) | 0 |
| Str faecal | 1 35 | 0<br>(+) | 1 5 | 1 05<br>(1 9) | 0<br>(2 0) | 1 5 | 0<br>(2 0) | 0<br>(1 25) | 0<br>(1 4) | 0 |
| S aureus phage type 42 D | 1 8<br>(2 2) | 1 8<br>(2 2) | 1 5 | 2 5<br>(3 2) | 2 5<br>(3 2) | 1 6 | 2 85<br>(3 2) | 2 3<br>(2 7) | 1 2<br>(1 3) | 0 |
| C albicans | 1 9<br>(2 9) | 1 8<br>(2 8) | 1 35 | 2 8<br>(4 0) | 3 0<br>(4 0) | 1 4 | 3 1<br>(4 25) | 2 3<br>(3 2) | 1 5<br>(2 0) | 2 2<br>(2 4) |
| Asp flav | 2 0<br>(2 6) | 1 9<br>(2 5) | 1 2 | 3 1<br>(3 8) | 3 1<br>(3 8) | 1 2 | 3 6<br>(4 35) | 2 55<br>(3 4) | 1 6 | |
| Asp fumigat | 3 0<br>(4 5) | 3 0<br>(4 5) | 1 5 | 4 1<br>(5 8) | 4 1<br>(5 6) | 1 5 | 4 1<br>(6 0) | | 2 1 | 2 3 |
| Tr mentagrophyt | 3 5 | 3 3 | 1 8 | 5 1 | 5 2 | 1 8 | 5 75 | 4 25 | 3 0 | 2 2 |

Cf the description of the method which precedes Table 1 The columns of figures in the table show the diameters of the inhibition zones in cm The figures not enclosed in brackets relate to the diameters of *absolute* inhibition zones the figures in brackets to the diameters of *absolute + relative* inhibition zones "Sal" stands for salicylanilide and "Cet" for *cetylpyridiniumchloride* "C" designates *preparations* made by the author (*Clausen*) + designates minor barely registrable relative zones 0 designates no absolute inhibition zones (+) minor, barely registrable, absolute inhibition zones The holes made in the media were 1 0 cm in diameter The ointment preparations numbered 1 6 are made by *Weiders Farmasoytiske* A/S Oslo

a) The same ointment with cetylpyridiniumchloride 0 1 per cent had the same effect With a pH value of 8 2 the effect was slightly less

*b)* The same ointment with cetylpyridiniumchloride 0 1 per cent had a distinctly weaker effect

c) The ointment also had an effect on the following fungi Crypt neoform 3 8 (5 5) Geotrich cand 4 8 (5 8), Asp niger 3 6 (4 1), Tr mentagrophytes (strain 2) 5 6

d) The ointment was also investigated in respect to its effect on the following two fungi Geotrich cand 3 4 (4 5) Tr mentagrophytes (strain 2) 4 0

FROM THE UNIVERSITY OF OSLO, NORWAY, INSTITUTE OF PHARMACY, DEPARTMENT OF BACTERIOLOGY. HEAD: OLE G. CLAUSEN, Ph.D.

# AN INVESTIGATION INTO THE BACTERIOSTATIC AND FUNGISTATIC EFFECTS OF CERTAIN CHEMICAL COMPOUNDS, PARTICULARLY WITH A VIEW TO THEIR POSSIBLE APPLICATION IN CHEMOTHERAPY

By

GUNNAR STEENSHOLT and OLE G. CLAUSEN

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As a part of a more broadly planned, systematic investigation into the antimicrobial effects of certain new, and also earlier synthesized but not investigated, chemical compounds (*Clausen* 1962, *Clausen & Kjeldgaard* 1962) on a variety of resistant and also important pathogenic bacterium and fungus strains, the authors of this paper have studied two pyridinealdehydes, three pyridinealdehyde thiosemicarbazones, three pyridinealdoximes, three picolylamines, 2,2'-pyridoin, and 2,2'-pyridil. Sulphamezathine and sulphamethizol were used as reference preparations in the bacteriostatic investigations, and fungizone "Squibb" in the fungistatic ones.

Of the substances referred to above, the 3-pyridinealdehyde thiosemicarbazones were made by one of the authors (*Steensholt*), while the others are known compounds, manufactured by *Dr. F. Raschig*, Chemische Fabrik, Ludwigshafen am Rhein. Nothing has been found in the literature regarding the effect on microbes of the substances forming the subject of this investigation.

## MEDIA AND METHODS

For details of the substances dealt with and the test microbes employed the reader is referred to the tabular survey on p. 328. The method employed was identical with the one previously described by *Clausen* (1962). It consisted in *bacteriostatic* investigation by means of a diffusion method in 10 per cent peptone-free horse blood agar (depth c. 0.7 cm, pH 7.4), made from Japanese agar, quality Kobe I), aqueous solutions of the substances being introduced into holes 1.0 cm in diameter

We wish to thank the department's assistants Mrs. *Eva Lovstad* and Mrs. *Kari Raugstad* for their great help in connection with the experiments.

SUMMARY

*A comparative microbiological investigation in vitro* of salicylanilide and certain other substances with an antimycotic effect, among these undecylenic acid and Amphotericin B, has shown that salicylanilide as sodium salt in aqueous dilutions between 10⁻² and $10^{-3}$ (in the case of *Amphotericin B, between ½ 10⁻² and ¹/₈ 10⁻²)* on the whole is by far superior to the other substances investigated in regard to bacteriostatic, but primarily fungistatic, effect

An attempt has been made to exploit in practice the good antimicrobial effect of salicylanilide by preparing and subjecting to microbiological investigation a number of new ointment compositions employing polyethyleneglycols as an ointment base, and containing salicylanilide, as well as combinations of salicylanilide and cetylpyridiniumchloride (= *Pyrisept "Weifa") as active constituents It was found that the pH* values of the ointment preparations were decisive in determining their effect *in vitro*

Polyethyleneglycol ointments with 2½ per cent salicylanilide and *with a pH value of between 8 2 and 9 5 proved to have very good anti*microbial effects, the optimum effect was attained at a pH value of 9 5 The addition of 0 2 per cent or 0 1 per cent cetylpyridiniumchloride had no appreciable influence on the effect at a pH in the range 8 2 to 8 8 *The fungistatic effects of the ointments were particularly good*

According to the method employed, the effects of these salicylanilide ointments were far superior to those of the preparation Decylene cream made up at a chemist's and those of Mycostatin ointment, manufactured by AB Bofors

Beyond this it must be left to dermatologists to assess the clinical effects of the salicylanilide ointment, and also to decide whether, with the relatively high pH value of c 8 8, it is sufficiently tolerable for skin application

REFERENCES

*Clausen O G* [illegible] Determination of the bacteriostatic [illegible] de and certain substituted salicylani[illegible] *solid media with and with*out horse [illegible] 950 1962

*Kjeldgaard K* [illegible] 1960

TABLE 2

*The Bacteriostatic and Fungistatic Effects of Certain Chemical Compounds in 10 2[1] Aqueous Solutions*

| Test microbes | 7<br>Pyridine 3 aldoxime pH 9 8 | 8<br>Pyridine 4 aldoxime pH 9 2 | 9<br>2 Picolyl amine 10 1 | 10<br>3-Picolyl amine pH 10 0 | 11<br>4 Picolyl amine pH 10 4 | 12<br>2 2 Pyridoin pH 7 9 | 13<br>2 2 Pyridil pH 7 0 |
|---|---|---|---|---|---|---|---|
| Ps aerug | 0<br>(1 2) | 0<br>(1 6) | 0<br>(1 4) | 0<br>(1 2) | 0<br>(1 05) | 0 | 0 |
| E coli | 0 | 0 | 0<br>(1 4) | 0<br>(1 2) | 0<br>(1 1) | 0 | 0 |
| S aureus | 0 | 0 | 0<br>(1 3) | 0 | 0<br>(1 2) | 0 | 0<br>(2 0) |
| Str faecal | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| C albicans | 0 | 0 | 1 7<br>(2 0) | 1 4 | 0<br>(1 2) | 0 | 0 |
| Asp fumigat | 0<br>(2 0)*<br>(2 9) | 0<br>(3 2)* | 2 4<br>(3 2) | 1 4<br>(1 8)*<br>(3 2) | 0<br>(2 4) | 0 | 0 |
| Tr mentagrophyt | 0<br>(+) | 0<br>(2 9) | 0<br>(2 25)* | 2 4<br>(4 5) | 0<br>(1 6) | 0 | 0 |

[1] For details of the solubility of the substances cf "Media and Methods" p 330

TABLE 1

*The Bacteriostatic and Fungistatic Effects of Certain Chemical Compounds in 10 2[1] Aqueous Solutions*

| Test microbes | 1<br>Pyridine 3-aldehyde<br>pH 5 9 | 2<br>Pyridine 4 aldehyde<br>pH 6 5 | 3<br>Pyridine 2 aldehyde thiosemicarbazone<br>pH 6 3 | 4<br>Pyridine 3-aldehyde thiosemicarbazone<br>pH 11 6 | 5<br>Pyridine 4 aldehyde thiosemicarbazone<br>pH 11 7 | 6<br>Pyridine 2 aldoxime[2]<br>10 2<br>pH 3 5 | 6<br>Pyridine 2 aldoxime[2]<br>10 3 |
|---|---|---|---|---|---|---|---|
| Ps aerug | 0<br>(1 7) | 1 6<br>(2 0) | 0 | 0 | 0 | 3 0<br>(3 6) | 0<br>(1 4) |
| E coli | 1 7<br>(2 6)<br>(3 2) | 3 5 | 0 | 0 | 0 | 2 3 | Not investigated |
| S aureus | 0<br>(1 7) | 1 9 | 0 | 0 | 0 | (3 0)<br>1 4 | Not investigated |
| Str faecal | (2 7)<br>0<br>(1 1) | (2 3)<br>0 | (1 5)<br>0 | (1 1)<br>1 1<br>(1 4) | 1 2 | (2 3)<br>1 3 | Not investigated |
| C albicans | 1 9<br>(2 4) | 2 5<br>(2 7) | 0 | 0 | 0 | 3 2<br>(3 7)+ | 0<br>(1 3) |
| Asp fumigat | 2 6<br>(3 5)<br>(3 9) | 3 1<br>(3 8) | 0 | 0 | 0<br>(1 5)+ | 3 4 | 0<br>(1 5) |
| Tr mentagrophyt | 2 5<br>(4 0)+ | 3 7<br>(4 2)+ | 0 | (1 8)<br>0<br>(4 0) | (2 9)<br>0<br>(3 2) | (4 1)<br>3 3<br>(4 4) | 0<br>(+) |

Cf the method described above

Absolute inhibition zones = zones totally devoid of growth

Relative = zones in which growth is distinctly inhibited to zones which are almost devoid of growth

The figures not in brackets relate to the diameters of absolute inhibition zones measured in cm, the figures in brackets relate to the diameters of absolute + relative inhibition zones also measured in cm

(+) = trace of relative inhibition zones

( ) = relative inhibition zones with very sparse growth

0 = no absolute inhibition zones

The holes made in media were 1 0 cm in diameter

[1] For details of the solubility of the substances cf Media and Methods p 330

[2] No effect was registrable in the dilution 10 4

Under the conditions set forth above the following substances could not give 1 per cent solutions in water, and accordingly were investigated in *saturated* solutions, with the pH values noted in the tables the thiosemicarbazones of pyridine-2-, pyridine-3-, and pyridine-4 aldehyde, pyridine 3 aldoxime and 2 2'-pyridil

Buffer solutions containing no antimicrobial substance and with pH values ranging from 6 0 up to 11 7 were investigated parallel with the other solutions

For details of the substances investigated and the result of the investigations, see the tabular survey and the Addendum

TABLE 4

*Fungizone Squibb*

| Test microbes | pH 7 4<br>1 2 10-2 | 10-3 | 10-4 | 10-5 | 10-6 |
|---|---|---|---|---|---|
| C. albicans | 2 15 | 2 15 | 2 15 | 2 0 | 1 8 |
| Asp fumigat | 2 6 | 2 2 | 2 0 | 1 95 | 1 65 |
| Tr mentagrophyt | 1 5 | 1 7 | 1 7 | 1 45 | 1 1<br>(1 25) |

Fungizone did not have a fungistatic effect on any of the fungus strains in the dilution 10 7

ADDENDUM

In addition to pyridine 2 aldoxime the following substances had an effect on certain of the test microbes in solutions with concentrations below 1 per cent, expressed in terms of the diameters of the inhibition zones Pyridine 3 aldehyde 0 01 per cent on Asp fumigat, relative zone 1 2 cm pyridine-4-aldehyde 0 1 per cent on C albicans, 1 4 cm, on Asp fumigat 1 5 cm and on Tr mentagrophyt 1 7 cm, all relative zones, 2 picolylamine 0 1 per cent on Asp fumigat, negligible trace of relative zone

In the following geometrical aqueous dilutions *pyridine 2-aldoxime* affected Ps aeruginosa and Tr mentagrophytes as shown below (As regards recording of the results, cf the tabular survey on p 328)

| *Ps aeruginosa* | | | | *Tr mentagrophytes* | | | |
|---|---|---|---|---|---|---|---|
| 1/100 | 1/200 | 1/400 | 1/800 | 1/100 | 1/200 | 1/400 | 1/800 |
| 2 8 | 2 0 | (1 6)* | (1 65) | 3 15 | 2 3 | (2 3) | (1 6) |
| (3 05)* | (2.3)* | (2 0) | | (4 4)* | (4 0) | | |
| (3 5) | (2 7) | | | | | | |

* Designates relative zones with very sparse growth

Pyridine-2-aldoxime caused the formation of a weak, but distinctly dark ring in the blood agar, encircling the holes containing solution Apart from this the substance appeared to be well compatible with blood in the medium as in the strongest concentration, 1 per cent, no more than a faint suggestion of a small haemolytic zone (c 1 3 cm in

made in the media, each of which in advance had been inoculated on the surface with a specifically diluted culture of the test microbe concerned, and subsequently dried For the *fungistatic* investigations the aforementioned medium with 1 per cent glucose was employed Large petri dishes some 13 5 cm in diameter (inside measurement) were used

The *bacterium inocula* were obtained by diluting 20-hour beef-peptone-broth cultures (cultivated at 37° C) in sterile, distilled water in the following proportions, expressed in ml inoculum per ml dilution Ps aerug 1 500, E coli 1 300, S aureus and Strept faecalis 1 200

The media were incubated for 20 hours at 37° C, after which the diameters of the inhibition zones were measured

The *fungus inocula* were obtained by suspending 10 day cultures, cultivated on Sabouraud agar at 25° C, in beef-peptone broth in mortars The Candida inoculum contained c 50 million living cells per ml and was diluted before use with sterile, distilled water in the ratio 1 40, whereas the inoculum with Asp fumigat, which contained 10–20 million living spores per ml, was diluted in the ratio 1 12 5 before use The inoculum with the Thrichophyton strain was obtained by suspending a 10-day Sabouraud-agar slant culture in 20 ml beef-peptone broth by means of a mortar The suspension was used undiluted as an inoculum The samples with C albicans as the test microbe were incubated for 20 hours at 37° C, those with Asp fumigat were incubated for 30 hours at the same temperature, while the Tr mentagrophytes samples were incubated at 25° C for 48 hours before the diameters of the inhibition zones were recorded The last mentioned fungus species was cultivated at a lower temperature to prevent the media drying up, as this fungus grows more slowly than the other two fungi at 37° C

TABLE 3

*The Bacteriostatic and Fungistatic Effects of the Reference Substances Used*

| Test microbes | Sulphamezathine pH 7 6 | | Sulphamethizol pH 2 | |
|---|---|---|---|---|
| | $10^{-2}$ | $10^{-3}$ | $10^{-2}$ | $10^{-3}$ |
| Ps aerug | 0 (1 7) | 0 | 0 (1 9) | 0 |
| E coli | 0 | 0 | 0 | 0 |
| S aureus | 3 0 | 2 0 (2 2) | 3 1 | 1 7 (1 9) |
| Str faecal | 0 | 0 | 0 | 0 |

None of the sulphonamides had any bacteriostatic effect in the dilution $10^{-4}$

The *substances* – *provided they were sufficiently soluble* – were investigated in 1 per cent aqueous solutions In certain cases they were also investigated in 0 1 per cent and 0 01 per cent solutions (cf Addendum) Where possible, heavily soluble substances were dissolved in 0 8 per cent NaOH, the pH value then being adjusted to the lowest possible level with the aid of HCl N/1

etter effect against C albicans, Asp fumigat, and Tr mentagrophytes han had an amphotericin B solution 0 5 per cent, which was the strongest investigated concentration of this antibiotic But in a concentration f 0 1 per cent amphotericin B showed about the same effect as in a 1 5 per cent solution, an effect which decreased only little even in very righ dilutions

The question of a possible chemotherapeutic effect of pyridine-2-aldoxime in vivo, however, will depend on the toxicity of the substance and on its tolerance by higher organisms Apart from specific antibiotics such as polymyxin and neomycin the authors, however, do not know of other chemical compounds possessing such excellent bacteriostatic effect against Ps aeruginosa as pyridine 2 aldoxime Its good effect against the other test bacteria, and on the fungus strains, gives rise to the possibility that pyridine-2-aldoxime can be used as a generally active chemotherapeutic for *local application, e g* in the form of powder solution, or ointment, provided that the toxicity and tolerance when the substance is applied locally prove satisfactory Pyridine 2 aldoxime must therefore first be tested on animals with regard to the aforesaid properties

Of the other substances investigated mention may be made of 2- and 3 picolylamine which had good fungistatic effects, both were more active than 4 picolylamine, and on the whole the effect decreased in the following order 2 3, 4 picolylamine the last-mentioned being the least active compound 2 2 pyridoin was inactive and 2 2 -pyridil nearly inactive

## SUMMARY

A determination of the bacteriostatic and fungistatic effects of certain pyridinealdehydes pyridinealdehyde thiosemicarbazones, pyridinealdoximes picolylamines, 2 2 pyridoin, and 2 2 pyridil has been carried out by the use of a diffusion method in peptone free 10 per cent horse blood agar The substances were investigated in solutions up to a maximum concentration of 1 per cent Sulphamezathine, sulphamethizol and amphotericin B were employed as reference compounds Pyridine 3 and pyridine 4 aldehyde were very effective against fungi, [illegible]

und being

were less

ter, 2, 3-,

and 4 picolylamine had a marked effect, principally against the fungus strains but with a distinctly decreasing tendency in the previously mentioned order 2 2 -pyridoin was ineffective and 2 2 -pyridil practically ineffective

Pyridine 2 aldoxime was in a 1 per cent solution on the whole the most effective of all the substances investigated Its exceptionally good bacteriostatic effect against Ps aeruginosa and also its good fungistatic effect in the presence of blood are noteworthy, and the possibili

diameter) round the edge of the hole was observed The effect of the substance on Ps aeruginosa was investigated in 1 per cent solutions with pH values of 9 5, 9 2 8 7, and 6 5 (the last-mentioned, nonadjusted pH) without it being possible to observe any change in the effect Nor could any change be discerned after a lapse of four weeks in a solution with a pH value of 8 7 after storage at 22° C in the dark

The buffer solutions containing no antimicrobial substance formed no inhibition zones in the pH range employed, 6 0 to 11 7

## DISCUSSION

Of the thirteen chemical compounds investigated pyridine-4-aldehyde generally proved distinctly more effective against both bacteria and fungi than pyridine-3-aldehyde The very good effects of both compounds on fungi are worthy of note Of the corresponding thiosemicarbazones pyridine-2-aldehyde thiosemicarbazone proved weakest and was practically devoid of demonstrable bacteriostatic and fungistatic effects, whereas the thiosemicarbazones of pyridine-3- and pyridine-4-aldehyde were clearly more effective, though it was impossible to differentiate between them in their effects they both proved substantially weaker in their effects than their corresponding pyridinealdehydes, a circumstance which may spring from their lesser solubility in water Unlike the aforementioned compounds, pyridine-2-aldoxime proved to possess both a considerably better bacteriostatic and fungistatic effect than pyridine-3- and pyridine-4-aldoxime, both of which were inactive against most bacteria and only moderately active against two of the fungus strains, and inactive against the third Pyridine-2-aldoxime was the most effective of all the substances investigated against both bacteria and fungi Particularly notable is the exceptional effectiveness of this substance on Ps aeruginosa, which is recognised as being one of the most chemo-resistant nonsporogenic bacteria Attention is also drawn to the Addendum, where the effect of pyridine-2-aldoxime in geometrical aqueous dilutions is recorded from 1 100 to 1 800 In the last-mentioned dilution and also in the dilution $10^{-3}$ the bacteriostatic effect on Ps aeruginosa is still distinctly registrable The reference substances sulphamezathine and sulphamethizol had only a slight effect on Ps aeruginosa in the dilution $10^{-2}$ (small, relative inhibition zones), and they were completely inactive against E coli and Strept faecalis in the same dilution Against S aureus, however, the sulphonamides were decidedly more effective than pyridine-2-aldoxime

Against the three pathogenic fungus strains investigated pyridine-2-aldoxime had a noteworthy good effect It is characteristic that a 0 5 per cent aqueous solution of amphotericin B (= Fungizone 'Squibb') had an appreciable weaker effect on Tr mentagrophytes than had an aqueous solution of pyridine 2-aldoxime in the same concentration In a 1 per cent aqueous solution the latter substance had a substantially

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# PRODUCTION OF ANTISERA TO NORMAL HUMAN ERYTHROCYTES COATED WITH INCOMPLETE SPECIFIC ANTIBODIES AND BOUND COMPLEMENT (EAC')

*By*

HARALD ORJASÆTER

Received 28 iv 62

Production of antiglobulin sera by injecting into suitable animals serologic specific materials, such as specific sensitized erythrocytes (I A) or antibody complement complexes (AC'), is not a usual procedure and has not been closely investigated, although it has been shown that potent antisera can be obtained this way (1, 2, 3, 6, 9)

These reports, however, show some contradictions concerning the specificity of the antiglobulin sera produced Antiglobulin sera are classified as 'anti gamma globulin", "anti-non gamma globulin" and broadspectrum' antisera (7)

*Van Loghem et al* (9) produced antisera to human red cells sensitized with antibodies of different kinds incomplete Rh antibodies as well as incomplete 'thermolabile cold antibodies It was found that these antisera could agglutinate cells sensitized with the different kinds of antibodies, i e, the antisera had a broad antiglobulin spectrum Similar results have been found by *Komninos & Aksoy* (2), who also injected the antibodies eluted from the red cells of patients suffering from auto immune haemolytic anaemia, and found no significant difference in the reactivity of these antisera against iso or auto antibodies

Some antibodies are known to fix complement (7) and there is some evidence that complement, or something associated with the complement activity, actually represents the materials against which the activity of the anti non gamma globulin" reagent is directed This is shown by *Rosenfield et al* (6), who produced antisera to specific precipitates containing bound human or guinea pig complement These antisera possessed strong anti AC' activity, but only weak reactions were observed against antibodies which did not fix complement

The author is greatly indebted to *Dr F Stratton Manchester* for reading the manuscript and making valuable suggestions

ties inherent in this compound as a chemotherapeutic are discussed above Pyridine-3- and pyridine-4-aldoxime had little bacteriostatic and fungistatic effect

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complete Lewis-antibodies and active human complement Maximum activity was shown up to a dilution of 1 in 20 There was no prozone phenomenon

TABLE 1

*The Anti Human Globulin Activity of four Rabbit Antisera*

| Antisera dil 1 10 No | Cell sensitized with anti $Le^b$ and active human complement | | |
|---|---|---|---|
| | O Le (a−b+) | (O Le (a−b−) | O Le (a+b−) |
| 1296 | +++ | — | — |
| 1297 | +++ | — | — |
| 1298 | +++ | — | — |
| 1299 | +++ | — | — |
| AHG 9 | ++(+) | - | — |
| 93 | (+) | (+) | |
| 1274 | (+) | (+) | |

Sera no 93 and 1274 are controls

Antiglobulin activity was observed against different complement fixing antibodies, such as incomplete cold anti-H, incomplete antibodies from a patient suffering from acquired haemolytic anaemia of the 'cold' type, and several Lewis antibodies On the other hand the AHG-mix had no or little activity against incomplete antibodies of different kinds which do not fix complement (Table 2)

TABLE 2

*Some Results from Testing of Cells Weakly Sensitized with Different Kinds of Antibody Using our Reagent (AHG mix) Compared with a Potent Broad Spectrum Antiglobulin Serum (AHG 9)*

| Cells weakly sensitized with * | AHG mix | AHG-9† |
|---|---|---|
| Cold incomplete anti H | ++ | +(+) |
| Incomplete antibody in serum from a patient with a h a "cold" type | ++(+) | +(+) |
| Incomplete anti $Le^a$ | ++ | +(+) |
| Incomplete anti $Le^b$ | ++(+) | ++ |
| Incomplete anti K† (not C fixing) | — | + |
| Incomplete anti D (not C fixing) | — | ++ |
| Incomplete anti $Fy^a$ (not C fixing) | — | ++ |

* Incubated with active complement

† Most anti K sera showed a weak activity of C fixing antibody

The antiglobulin activity against cells sensitized with incomplete anti-$Le^b$ with bound complement is further demonstrated in Table 3, where the activity is compared with that of two known sera, the one our selected broadspectrum reagent, the other a commercial "anti-non-gamma-globulin" reagent

Examples of the antiglobulin activity against high-titred antibodies which do not fix ... Table 4 A weak activity ... This activity, however, was ... to the reagent (Gamma-

Antisera with mostly "anti-non-gamma-globulin" activity were also obtained by *Gold & Lockyer* (1), who injected rabbits with formol-treated human red cells coated with "incomplete" anti A + complement

The object of this work was to investigate the production of anti globulin sera by immunization with antibody complement complexes (AC′) as well as to study further the specificity of these antisera It was shown that even weak incomplete antibodies with bound complement may elicit the formation of potent anti-AC′ sera

## MATERIALS AND METHODS

Four rabbits were immunized by intravenous injections of antibody coated human red cells of group O One ml of a 25 per cent cell suspension was given to each at intervals of 5 7 days in a series of 10 injections This procedure was repeated twice at about 6 months interval One week after the last injection in each series the rabbits were bled In the first series the cells were sensitized with very weak anti Jka (traces) and active human complement In the second and third series immunization was performed with red cells sensitized with incomplete anti Lea and anti Leb respectively and active human complement These antibodies were also weak with an antiglobulin titre of 16 or less In order to make sure that the cells had bound complement the sensitization was performed in two steps first with inactivated antisera to make FA and the ... (production of FAC′) After each step ... dspectrum antiglobulin reagent which ... ie positive when complement was absorbed (with FAC′)

The rabbit sera were inactivated by heating to 56° C for 30 min and absorbed at 4° C with human red cells of group A B O and AB Six to ten absorptions with an equal volume of packed red cells were required to remove the agglutinins active against human red cells

As a control two sera from rabbits injected with unsensitized human red cells were treated in the same way These rabbits had been injected several times in order to produce anti M or anti N and had given a very good antibody response

A potent broadspectrum antiglobulin reagent (AHG 9) was used for comparison of the antiglobulin activity of the produced antisera This reagent was produced in our laboratory by injecting whole human serum into rabbits It has proved to react very well with cells sensitized to complement fixing antibodies and bound complement as well as with antibodies which do not fix complement The reagent was selected among several other antiglobulin sera produced in our laboratory because of its strong activity against cells coated with antibodies and bound complement

## RESULTS

There was no anti human globulin activity in the rabbit sera collected after the first injection series with Kidd-sensitized cells After the second series with Le(a+) sensitized erythrocytes, a weak activity was noticed and the activity was much stronger after the last series with Le(b+)-sensitized cells As shown in Table I active antiglobulin sera then had been formed in all rabbits The two control sera did not show any specific antiglobulin activity

Further investigations were carried out using a mixture of these four sera (AHG-mix) The mixture showed antiglobulin activity up to a dilution of 1 in 320 when tested against red cells sensitized with in-

globulin 12 per cent "Kabi") in quantities which did not affect the reaction with complement fixing antibodies. Addition of whole-human serum suppressed all anti-globulin activity.

## DISCUSSION

By immunization of four rabbits with antibody complement coated human red cells (EAC'), antisera were formed which showed high activity against cells sensitized with antibody-complement complexes (AC'). The two control sera prepared with unsensitized cells did not show this activity. This indicates that the immune response must be due to the coating of the sensitized erythrocytes. The finding that the injection of uncoated normal human erythrocytes fail to form antiglobulin serum is supported by the findings of *Rose et al.* (5), who found no antiglobulin activity against human serum constituentes after immunization with haemolysates of normal human red cells.

The coating of the injected erythrocytes consisted of antibody-complement complexes (AC'). The antisera produced by immunization with these complexes, as shown in table 2, 3 and 4, had a specificity which reasonably may be designated 'anti-AC'' and no or little activity against antibodies which do not fix complement. By testing Lewis sensitized cells no activity was found against the antibodies before complement was absorbed. Hence, evidence seems to have been established that these antisera predominantly have anti C' specificity, as pointed out by *Stratton* (8). This is in fair accordance with the findings of *Rosenfield et al.* (6), who immunized with complement bound to specific precipitates, as well as with findings by *Gold & Lockyer* (1), who used complement bound to formol-treated and antibody coated human red cells, in either case most of the activity seemed to be directed against the antibody-complement complexes (AC') with evidence of anti C' specificity.

The injected erythrocytes were sensitized using very weak incomplete antibodies and active complement. Thus, even weak antibodies may absorb a sufficient amount of complement to give a satisfactory immune response.

It seems as if immunization with antibody-complement complexes (AC') in general elicit the formation of antisera of the type anti-AC', the specificity of which is predominantly anti C' and, as shown by *Rosenfield et al.* (6), the specificity also depends on the species specificity of the complement.

## SUMMARY

This investigation confirms previous observations that injection of antibody coated human red cells into rabbits can stimulate the f...

... AC' com-

TABLE 3

*The Results of Titrations Showing the Anti 4C Activity of our Reagent (AHG mix) Compared with two Potent Antiglobulin Sera Possessing Good Anti Non Gamma Globulin Activity*
*(The Reactions Performed as two Stage Antiglobulin Tests (4))*

| Cells | AHG sera* | Dilution of the sensitizing sera | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
| Le(b+) sens with act C | AHG mix | +++ | +++ | +++ | ++ | ++ | ++ | + | + | — |
| | AHG 9 | +++ | +++ | +++ | ++ | ++ | + | + | (+) | — |
| | Comm † | +++ | +++ | ++ | ++ | + | + | (+) | — | — |

* Sera were used in optimal dilutions
† A commercial anti non gamma globulin serum

TABLE 4

*Results of Titrations Showing the Anti Gamma Globulin Activity of our Reagent (AHG mix) Compared with a Potent Broadspectrum Antiglobulin Serum*

| Cells | AHG sera* | Dilutions of sensitizing sera | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:5 | 1:10 | 1:20 | 1:40 | 1:80 | 1:160 | 1/320 | 1:640 | 1:1280 | 1:2560 |
| Rh sens | AHG mix | ++ | ++ | + | — | — | — | — | — | — | — |
| | AHG 9 | ++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + |
| Fy(a+) sens | AHG mix | + | (+) | — | — | — | — | — | — | — | — |
| | AHG 9 | ++ | +++ | +++ | +++ | +++ | ++ | + | — | — | — |

* Sera used in optimal dilutions

AUS STATENS SERUMINSTITUT (INTERNATIONALE SALMONELLA- UND ESCHERICHIA-CENTRALE) KOPENHAGEN UND DER MEDIZINAL-UNTERSUCHUNGSANSTALT AM HYGIENISCHEN INSTITUT DER FREIEN UND HANSESTADT HAMBURG (LEITER: PROF. DR. MED. S. WINKLE)

# ZUR VEREINFACHUNG DER SEROLOGISCHEN SALMONELLA-DIAGNOSE

Von

F. KAUFFMANN und R. ROHDE

Eingegangen 9. V. 62

In einer vorhergehenden Mitteilung „*Eine Vereinfachung der serologischen Arizona-Diagnose*" schlugen wir vor, die *Arizona-species* mit Hilfe des vereinfachten *Kauffmann-White-Schemas* (K.-W.-Schema) zu diagnostizieren. In konsequenter Fortführung dieses Vorgehens schlagen wir jetzt vor, alle *neuen Salmonella-species* des *sub-genus* II mit Hilfe des vereinfachten K.-W.-Schemas zu diagnostizieren. Diese *species* sind mit den *Arizona-species* (= *Salmonella sub-genus* III) sehr nahe verwandt, verflüssigen Gelatine und verhalten sich in den organischen Säuren einheitlich, speziell ergeben sie einen positiven Malonat-Test. Auch in klinischer und epidemiologischer Hinsicht spielen die Angehörigen dieser beiden *sub-genera* eine ähnliche Rolle, sodass eine nähere Diagnose mit Hilfe des originalen K.-W.-Schemas im allgemeinen nicht nötig ist. Sollte es sich aber in speziellen Fällen bei gehäuftem Auftreten als wünschenswert erweisen, eine genauere Diagnose zu haben, so kann man natürlich jeder Zeit das originale K.-W.-Schema anwenden.

In allen denjenigen Fällen aber, in denen die vereinfachte Diagnose gestellt ist, wird nur die Antigenformel angegeben und dem Einsender mitgeteilt: *Salmonella sub-genus* II (unter Hinzufügung der Formel).

Betreffs der notwendigen Seren sei auf das Buch von *F. Kauffmann* „*Die Bakteriologie der Salmonella-Species*" (Seite 126–128) sowie auf die dort angegebene Literatur verwiesen.

Ferner möchten wir vorschlagen, alle *neuen species* des *sub-genus* I, welche die H-Antigenkomplexe G und L enthalten, nach dem vereinfachten K.-W.-Schema zu diagnostizieren. Hierdurch wird eine erhebliche Vereinfachung erreicht, da gerade die nähere Bestimmung dieser Antigenkomplexe zu Schwierigkeiten führt. Die praktische Erfahrung hat gezeigt, dass die im originalen K.-W.-Schema durchgeführte Aufteilung der G- und L-Komplexe in zahlreiche Partialantigene die Grenze der praktischen Zuverlässigkeit und Reproduzierbarkeit bereits überschritten hat. Es ist daher vorzuziehen, auf die so erreichte hohe Dif-

plex However, the antibodies formed may be predominantly of anti C specificity, and it is pointed out that even very weak antibodies may bind a sufficient amount of complement to give the red cells this special antigenic property

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# ZUR VEREINFACHUNG DER SEROLOGISCHEN SALMONELLA DIAGNOSE

Von

F KAUFFMANN und R ROHDE

Eingegangen 9 X 62

In einer vorhergehenden Mitteilung *Eine Vereinfachung der serologischen Arizona Diagnose* schlugen wir vor die *Arizona species* mit Hilfe des vereinfachten *Kauffmann White Schemas* (K W Schema) zu diagnostizieren In konsequenter Fortfuhrung dieses Vorgehens schlagen wir jetzt vor alle *neuen Salmonella species* des *sub genus* II mit Hilfe des vereinfachten K W Schemas zu diagnostizieren Diese *species* sind mit den *Arizona species* (— *Salmonella sub genus* III) sehr nahe verwandt verflussigen Gelatine und verhalten sich in den organischen Sauren einheitlich speziell ergeben sie einen positiven Malonat Test Auch in klinischer und epidemiologischer Hinsicht spielen die Angehorigen dieser beiden *sub genera* eine ahnliche Rolle so dass eine nahere Diagnose mit Hilfe des originalen K W Schemas im allgemeinen nicht notig ist Sollte es sich aber in speziellen Fallen bei gehauftem Auftreten als wunschenswert erweisen eine genauere Diagnose zu haben so kann man naturlich jeder Zeit das originale K W Schema anwenden

In allen denjenigen Fallen aber in denen die vereinfachte Diagnose gestellt ist wird nur die Antigenformel angegeben und dem Einsender mitgeteilt *Salmonella sub genus* II (unter Hinzufugung der Formel)

Betreffs der notwendigen Seren sei auf das Buch von *F Kauffmann Die Bakteriologie der Salmonella Species* (Seite 126–128) sowie auf die dort angegebene Literatur verwiesen

Ferner mochten wir vorschlagen alle *neuen species* des *sub genus* I welche die H Antigenkomplexe G und I enthalten nach dem vereinfachten K W Schema zu diagnostizieren Hierdurch wird eine erhebliche Vereinfachung erreicht da gerade die nahere Bestimmung dieser Antigenkomplexe zu Schwierigkeiten fuhrt Die praktische Erfahrung hat gezeigt dass die im originalen K W S h —
lung der G und I Komplexe in zahl
der praktischen Zuverlassigkeit und
schritten hat Es ist daher vorzuziehen auf die so erreichte hohe Dif

ferenzierungs-Möglichkeit zu verzichten und diese Antigenkomplexe nur mit G oder L zu bezeichnen. Je mehr wir die einzelnen Antigenkomplexe vereinfachen, um so sicherere und übereinstimmendere Diagnosen erhalten wir, jedoch müssen wir dann die geringere Differenzierungs-Möglichkeit in Kauf nehmen. Es ist unmöglich, beide Vorteile: Einfachheit der Diagnose und grosse Differenzierungs-Möglichkeit miteinander zu vereinen.

Schliesslich möchten wir zur Diskussion stellen, in Zukunft alle *neuen Salmonella-species* des *sub-genus* I nur nach dem vereinfachten K.-W.-Schema zu diagnostizieren, abgesehen von besonderen, epidemiologisch wichtigen Fällen. Wird zum Beispiel eine neue *Salmonella-species* aus einem Salamander nur einmal isoliert, so ist es überflüssig, diese Kultur in das originale K.-W.-Schema einzufügen.

## ZUSAMMENFASSUNG

1. Es wird vorgeschlagen, alle *neuen species* des *Salmonella subgenus* II nach dem vereinfachten *Kauffmann-White-Schema* zu diagnostizieren.

2. Es wird vorgeschlagen, alle *neuen species* des *Salmonella subgenus* I mit den H-Antigenkomplexen G und L nach dem vereinfachten *Kauffmann-White-Schema* zu diagnostizieren.

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AUS STATENS SERUMINSTITUT (INTERNATIONALE SALMONELLA UND ESCHERICHIA CENTRALE) KOPENHAGEN

# ZUR SEROLOGIE DER SALMONELLA O GRUPPEN 28, 40, 45 UND 47

Von

F KAUFFMANN und A PETERSEN

Eingegangen 11 V 62

Da die O Gruppen 28, 40, 45 und 47 komplex gebaut sind und diagnostische Seren zur Bestimmung dieser Gruppen polyvalent sein sollen, so wird im folgenden hierauf naher eingegangen, da diese Verhältnisse nicht allgemein bekannt sind Es kommt hinzu, dass im Laufe der letzten Jahre zahlreiche *species* dieser O Gruppen gefunden wurden, ohne dass in allen Fällen eine Einordnung in die betreffenden O Untergruppen vorgenommen wurde Es sei jedoch erwahnt, dass diese 4 O Gruppen im *Kauffmann White-Schema* nach wie vor mit 28 40, 45, resp 47 bezeichnet werden, sodass also die betreffenden O Untergruppen hierin nicht berucksichtigt werden

## DIE O GRUPPE 28

Die *type species* dieser O Gruppe, *Salmonella tel aviv*, wurde von *F Kauffmann* (1) im Jahre 1940 publiziert, wahrend *Salmonella dakar*, mit abweichenden O-Antigenen, von *H Darrasse & L Le Minor* 1955 beschrieben wurde Die serologischen Unterschiede zwischen den O Antigenen dieser beiden *species* konnen durch folgende Formeln ausgedruckt werden

$$S\ tel\ aviv = 28_1, 28_2$$
$$S\ dakar = 28_1, 28_3$$

Zur Diagnose dieser beiden O Untergruppen sind also 2 Faktor Seren $28_2$ und $28_3$ notwendig und werden durch Absorption der O Seren von *S tel-aviv* mit *S dakar* resp von *S dakar* mit *S tel-aviv* hergestellt Absorbiert man diese O Seren in der Verdünnung 1 2 oder 1 5 so erhält man Faktor

tion die Diff

mittelt dass zur Untergruppe $28_1$, $28_2$ gehoren

*S abadina*
*S adamstown*
*S ank*
*S ashanti*
*S babelsberg*
*S bokanjac*

ferenzierungs-Moglichkeit zu verzichten und diese Antigenkomplexe nur mit G oder L zu bezeichnen Je mehr wir die einzelnen Antigenkomplexe vereinfachen um so sicherere und ubereinstimmendere Diagnosen erhalten wir, jedoch mussen wir dann die geringere Differenzierungs Moglichkeit in Kauf nehmen Es ist unmoglich, beide Vorteile Einfachheit der Diagnose und grosse Differenzierungs Moglichkeit miteinander zu vereinen

Schliesslich mochten wir zur Diskussion stellen, in Zukunft alle *neuen Salmonella species* des *sub-genus* I nur nach dem vereinfachten K W Schema zu diagnostizieren, abgesehen von besonderen epidemiologisch wichtigen Fallen Wird zum Beispiel eine neue *Salmonella species* aus einem Salamander nur einmal isoliert so ist es uberflussig diese Kultur in das originale K W Schema einzufugen

## ZUSAMMENFASSUNG

1 Es wird vorgeschlagen, alle *neuen species* des *Salmonella sub genus* II nach dem vereinfachten *Kauffmann White Schema* zu diagnostizieren

2 Es wird vorgeschlagen, alle *neuen species* des *Salmonella sub genus* I mit den H Antigenkomplexen G und L nach dem vereinfachten *Kauffmann-White Schema* zu diagnostizieren

## LITERATUR

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*Kauffmann F & Rohde R* Eine Vereinfachung der serologischen Arizona Diagnose Acta path et microbiol scandinav 54 473 478 1962

oder *S dakar* resp mit beiden Kulturen absorbiert Aus der Tabelle 2 geht hervor dass *S aderike* sowohl von *S tel aviv* als auch von *S dakar* verschieden ist Nach Absorption des O Serum von *S aderike* mit *S tel aviv* + *S dakar* blieben noch Agglutinine fur *S aderike* im Serum zuruck

## DIE O GRUPPE 40

Die *type-species* dieser O Gruppe *Salmonella riogrande* wurde von *P R Edwards A B Moran J Watt & Th De Capito* im Jahre 1950 publiziert wahrend *Salmonella bulawayo* mit abweichenden O Antigenen von *F Kauffmann & J Deom* 1939 beschrieben wurde Diese *species* wurde zunachst als neue O Gruppe = O 49 aufgefasst aber 1959 von *F Kauffmann* (2) mit der O Gruppe 40 vereint

A new type *S bukavu* — 1 40 1 z 5 1 5 is closely related to both O groups 40 and 49 and would cause great difficulties in differentiating between 40 and 49 *Salmonella bukavu* wurde von *F van Oye P Vassiliadis P Janssen & G van Looy* 1959 publiziert

Die serologischen Unterschiede zwischen den O Antigenen dieser 3 *species* konnen durch folgende Formeln ausgedruckt werden

*S riogrande* — $40_1$ 40
*S bulawayo* = 1 $40_1$ $40_3$
*S bukavu* = 1 $40_1$ 40

Geringe Unterschiede die zwischen *S riogrande* und *S bukavu* bestehen sind in diesen Formeln nicht berucksichtigt (siehe Tabelle 3) Zur Diagnose dieser beiden O Untergruppen sind also 2 Faktor Seren 40 und $40_3$ notwendig und werden durch Absorption der O Seren von *S riogrande* mit *S bulawayo* resp von *S bulawayo* mit *S riogrande* + *S bukavu* hergestellt Absorbiert man diese O Seren in der Verdunnung 1 2 oder 1 5 so erhalt man Faktor Seren mit denen man bereits in der Objektglas Agglutination die Differential Diagnose stellen kann Auf diese Weise wurde ermittelt dass folgende 20 *species* zur Untergruppe 40 $40_2$ gehoren

*S allandale*
*S benguella*
*S boksburg*
*S bukavu*
*S degania*
*S driffield*
*S dural*
*S fandran*
*S greiz*
*S johannesburg*
*S karamoja*
*S millesi*
*S nowawes*
*S omifisan*
*S riogrande*
*S santhiaba*
*S shikmonah*
*S springs*
*S suarez*
*S tilene*

S. brisbane
S. ceres
S. chicago
S. ezra
S. friedrichsfelde
S. guildford
S. halle
S. hermannswerder
S. idala
S. kaltenhausen
S. kibusi
S. kuessel
S. langford
S. leoben
S. luckenwalde
S. mocamedes
S. moero
S. moroto
S. mundonobo
S. nashua
S. nima
S. ona
S. patience
S. pomona
S. seattle
S. solna
S. taunton
S. techimani
S. tel-aviv
S. umbilo
S. vinohrady
S. vitkin
S. wedding = 28 : c : e,n,$z_{15}$
Salmonella 28 : $z_4$,$z_{23}$ : –

TABELLE 1
*O Gruppe 28*

| Kultur | O Seren | | | |
|---|---|---|---|---|
| | S. tel aviv | | S. dakar | |
| | Nicht absorb. | Absorb. mit S. dakar | Nicht absorb. | Absorb. mit S. tel aviv |
| S. tel aviv | 320 | 256 | 20 | 0 |
| S. dakar | 20 | 0 | 320 | 256 |
| S. aderike | 160 | 128 | 160 | 64 |

Die nicht absorbierten Seren wurden ab 1 : 10 angesetzt, die absorbierten Seren ab 1 : 4, da sie in der Verdünnung 1 : 2 absorbiert wurden. 0 = negativ in 1 : 4.

TABELLE 2
*S. aderike O Serum*

| Kultur | Nicht absorb. | Absorbiert mit | | |
|---|---|---|---|---|
| | | S. tel aviv | S. dakar | S. tel aviv + S. dakar |
| S. tel aviv | 320 | 0 | 80 | 0 |
| S. dakar | 320 | 160 | 0 | 0 |
| S. aderike | 640 | 160 | 320 | 80 |

0 = negativ in 1 : 10.

Zur Untergruppe $28_1$, $28_3$ gehört bisher nur *S. dakar*.

Eine neue *species S. aderike* = 28 : $z_{38}$ : –, von *J. Taylor* erhalten, verhielt sich abweichend, da sie von beiden Faktor-Seren $28_2$ und $28_3$ agglutiniert wurde (siehe Tabelle 1). Um dieses eingehender zu untersuchen, wurde ein O Serum von *S. aderike* hergestellt und mit *S. tel-aviv*

& *Sen* (1960) erwähnt Die serologischen Unterschiede zwischen diesen beiden *species* können durch folgende Formeln ausgedrückt werden

$$S\ deversoir = 45_1, 45_2$$
$$S\ dugbe = 45_1, 45_3$$

Zur Diagnose dieser beiden O Untergruppen sind also 2 Faktor-Seren $45_2$ und $45_3$ notwendig und werden durch Absorption der O Seren von *S deversoir* mit *S dugbe*, resp von *S dugbe* mit *S deversoir* hergestellt Absorbiert man diese O Seren in der Verdünnung 1 2 oder 1 5, so erhält man Faktor Seren, mit denen man bereits in der Objektglas-Agglutination die Differential-Diagnose stellen kann

Auf diese Weise wurde festgestellt, dass folgende 5 *species* zur $45_1$, $45_2$-Untergruppe und folgende 5 *species* zur $45_1, 45_3$ Untergruppe gehören

| $45_1, 45_2$ | $45_1, 45_3$ |
|---|---|
| *S apapa* | *S dugbe* |
| *S deversoir* | *S ejeda* = 45 a $z_{10}$ |
| *S jodhpur* | *S karachi* |
| *S windhoek* | *S suelldorf* = 45 f,g – |
| *Salmonella* 45 g m | *Salmonella* 45 a e,n,x |

TABELLE 4
O-Gruppe 45

| Kultur | O-Seren | | | |
|---|---|---|---|---|
| | S deversoir | | S dugbe | |
| | Nicht absorb | Absorb mit S dugbe | Nicht absorb | Absorb mit S deversoir |
| *S deversoir* | 640 | 512 | 320 | 0 |
| *S dugbe* | 160 | 0 | 320 | 128 |
| *S perinet* | 160 | 32 | 320 | 32 |

Die nicht absorbierten Seren wurden ab 1 10 angesetzt die absorbierten Seren ab 1 4 da sie in der Verdünnung 1 2 absorbiert wurden 0 = negativ in 1 4

TABELLE 5
*S perinet O-Serum*

| Kultur | S perinet O-Serum | | |
|---|---|---|---|
| | Nicht absorb | Absorbiert mit | |
| | | S deversoir | S dugbe |
| *S deversoir* | 40 | 0 | 20 |
| *S dugbe* | 40 | 10 | 0 |
| *S perinet* | 640 | 640 | 640 |

Zur Untergruppe $40_1$, $40_3$ gehören folgende 3 *species*: *S. alsterdorf* = 1,40 g,m,t –, von *R. Rohde* erhalten, *S. bern* und *S. bulawayo*. *S. bern* wurde von *F. Kauffmann, H. Fey & F. Steck* 1960 beschrieben, doch wurde damals eine nähere Analyse der O-Antigene nicht ausgeführt. Während *S. bern* in der Objektglas-Agglutination nur vom $40_3$-Serum agglutiniert wurde, ergab die Reagenzglas-Agglutination bei sehr starker Reaktion im $40_3$-Serum (1:256) auch noch eine schwächere Reaktion (1:32) im $40_1$-Serum.

TABELLE 3
*O-Gruppe 40*
*S. riogrande O-Serum*

| Kultur | Nicht absorb. | Absorbiert mit | | |
|---|---|---|---|---|
| | | S. bulawayo | S. bukavu | S. bukavu + S. bulawayo |
| *S. riogrande* | 320 | 256 | 8 | 8 |
| *S. bulawayo* | 10 | 0 | 0 | 0 |
| *S. bukavu* | 80 | 64 | 0 | 0 |

*S. bulawayo O-Serum*

| | Nicht absorb. | Absorbiert mit | | |
|---|---|---|---|---|
| | | S. riogrande + S. senftenberg | S. bukavu | S. bukavu + S. riogrande |
| *S. riogrande* | 80 | 0 | 4 | 0 |
| *S. bulawayo* | 160 | 128 | 128 | 128 |
| *S. bukavu* | 80 | 16 | 0 | 0 |

*S. bukavu O-Serum*

| | Nicht absorb. | Absorbiert mit | | |
|---|---|---|---|---|
| | | S. riogrande + S. senftenberg | S. bulawayo | S. bulawayo + S. riogrande |
| *S. riogrande* | 320 | 0 | 256 | 0 |
| *S. bulawayo* | 20 | 0 | 0 | 0 |
| *S. bukavu* | 320 | 8 | 256 | 8 |

Die nicht absorbierten Seren wurden ab 1:10 angesetzt, die absorbierten Seren ab 1:4, da sie in der Verdünnung 1:2 absorbiert wurden. 0 = negativ in 1:4.

## DIE O-GRUPPE 45

Die „*type-species*“ dieser O-Gruppe 45, *Salmonella deversoir*, wurde von *Watkins, Douglas & Taylor* 1955 beschrieben, während eine hiervon abweichende *species*, *Salmonella dugbe*, im Jahre 1956 von *J. Taylor* erhalten wurde. Diese *species* ist in der Veröffentlichung von *Collard*

TABELLE 6

*O Gruppe 47*

| Kultur | O Seren | | | |
|---|---|---|---|---|
| | S bergen | | S kaolack | |
| | Nicht absorb | Absorb mit S kaolack | Nicht absorb | Absorb mit S bergen |
| *S bergen* | 640 | 512 | 80 | 0 |
| *S kaolack* | 40 | 0 | 1280 | 256 |
| *S bergen* | 80 | 0 | 1280 | 256 |

Die nicht absorbierten Seren wurden ab 1 10 angesetzt, die absorbierten Seren ab 1 4 da sie in der Verdunnung 1 2 absorbiert wurden 0 = negativ in 1 . 4

In der taglichen Diagnose von *Salmonella*-Kulturen werden zur Bestimmung dieser 4 O-Gruppen 28, 40, 45 und 47 polyvalente O Seren benutzt Das O 28 Serum ist ein polyvalentes Serum von *S tel-aviv* + *S dakar*, das O 40 Serum ist ein polyvalentes Serum von *S riogrande* + *S bulawayo*, das O 45 Serum ist ein polyvalentes Serum von *S deversoir* + *S dugbe*, wahrend das O 47 Serum ein polyvalentes Serum von *S bergen* + *S kaolack* ist

DISKUSSION

Die Haufigkeit der einzelnen O-Untergruppen innerhalb der 4 O-Gruppen 28, 40, 45 und 47 ist verschieden So gehoren in der O Gruppe 28 zur $28_1$ $28_2$-Untergruppe 40 *species* (*S tel-aviv* und andere), während zur $28_1$, $28_3$-Untergruppe bisher nur *S dakar* gehort Abweichend hiervon verhalt sich *S aderike*, da sie von beiden Faktor-Seren $28_2$ und $28_3$ agglutiniert wird, sodass die O Formel mit $28_1$, $28_2$, $28_3$ angegeben werden kann In der praktischen Diagnose dieser Kulturen wird jedoch auf die einzelnen Partialantigene keine Rucksicht genommen, da alle vereinfacht als O 28 diagnostiziert werden, und zwar mit Hilfe eines polyvalenten Immunserum von *S tel-aviv* + *S dakar*

Innerhalb der O-Gruppe 40 gehoren 20 *species* (*S riogrande* und andere) zur $40_1$, $40_2$-Untergruppe, während zur $40_1$, $40_3$-Untergruppe 3 *species* (*S bulawayo*, *S bern* und *S alsterdorf*) gehoren In der praktischen Diagnose dieser Kulturen wird aber auf die einzelnen Partialantigene keine Rucksicht genommen, da alle vereinfacht als O 40 diagnostiziert werden, und zwar mit Hilfe eines polyvalenten Immunserum von *S riogrande* + *S bulawayo*, das mit *S urbana* (= O 30) absorbiert ist

In ahnlicher Weise liegen die Verhältnisse in der O Gruppe 45, bei der 5 *species* zur O-Untergruppe $45_1$, $45_2$ (*S deversoir*) und 5 *species* zur O-Untergruppe $45_1$, $45_3$ (*S dugbe*) gehoren Auch hier weicht eine neue *species*, *S perinet*, dadurch von den übrigen Vertretern dieser Gruppe ab, dass sie von beiden Faktor-Seren agglutiniert wird Wie die mit dem O-Serum von *S perinet* ausgeführten Absorptions-Versuche

Eine weitere, von *L. Le Minor* erhaltene *species*, *S. perinet* =45 mit e,n,x,$z_{15}$ wurde von beiden Faktor-Seren 45 und $45_3$ agglutiniert (siehe Tabelle 4). Zwecks näherer Untersuchung wurde ein O-Serum von *S. perinet* hergestellt und mit *S. deversoir* resp. *S. dugbe* absorbiert. Die Tabelle 5 zeigt, dass nach Absorption mit diesen Kulturen noch starke Agglutinine für *S. perinet* im Serum zurückblieben.

### DIE O-GRUPPE 47

Die „*type-species*" dieser O-Gruppe 47, *Salmonella bergen*, wurde von *F. Kauffmann, N. Saervold, F. Kristiansen & S. D. Henriksen* 1953 beschrieben, während eine hiervon abweichende *species* *S. kaolack* im Jahre 1956 von *L. Le Minor, H. Darrasse & R. Nazaud* publiziert wurde. Die serologischen Unterschiede zwischen diesen beiden *species* können durch folgende Formeln ausgedrückt werden:

$$S.\ bergen = 47_1, 47_2$$
$$S.\ kaolack = 47_1, 47_3$$

Zur Diagnose dieser beiden O-Untergruppen sind also 2 Faktor-Seren $47_2$ und $47_3$ notwendig und werden durch Absorption der O-Seren von *S. bergen* mit *S. kaolack*, resp. von *S. kaolack* mit *S. bergen* hergestellt. Absorbiert man diese O-Seren in der Verdünnung 1:2 oder 1:5, so erhält man Faktor-Seren, mit denen man bereits in der Objektglas-Agglutination die Differential-Diagnose stellen kann. Auf diese Weise wurde festgestellt, dass folgende 2 *species* zur $47_1$, $47_2$ Untergruppe gehören: *S. bere* und *S. bergen*.

Im Gegensatz hierzu gehören folgende 11 *species* zur $47_1$, $47_3$ Untergruppe:

- *S. bilthoven*
- *S. bootle*
- *S. kaolack*
- *S. luke*
- *S. lyon*
- *S. mount pleasant*
- *S. phoenix*
- *S. quimbamba*
- *S. quinhon*
- *S. saka*
- *S. teshie*

Ferner gehört zu dieser Untergruppe $47_1$, $47_3$ eine im Jahre 1958 von *J. Taylor* erhaltene Kultur von „*S. bergen*" mit der Formel $47_1$, $47_3$ : i : e,n,$z_{15}$, die also das H-Antigen der originalen *S. bergen*-Kultur = $47_1$, $47_2$ : i : e,n,$z_{15}$ besitzt. In Wirklichkeit handelt es sich also hierbei um eine neue *species*, doch wurde sie als „*S. bergen*" bezeichnet, da im *Kauffmann-White-Schema* die beiden O-Untergruppen nicht angegeben sind (siehe Tabelle 6).

*Kauffmann F & Deom J* "A new Salmonella type (*Salmonella bulawayo*) = (1), [illegible]

[illegible]

*Minor L Le, Darrasse, H & Vazaud R* "Un nouveau sérotype de Salmonella S *kaolack* (47 z-l 6) Annal Inst Pasteur, *91* 400, 1956

*Oye E van Vassiliadis, P, Janssen, P & Looy G van* "Une nouvelle Salmonella isolee au Congo Belge *S bukavu* (1 40 l $z_{28}$ 1 5)" Annal Inst Pasteur, *96* 370, 1959

*Watkins J F Douglas S H & Taylor, J* "A new Salmonella serotype (*Salmonella deversoir*) isolated from human faeces" Internat Bull Bacter Nomenclat & Taxon, *5* 5, 1955

zeigen, enthalt diese *species* einen stark entwickelten Sonderfaktor Fur die praktische Diagnose aller, dieser Kulturen genugt aber ein polyvalentes O-Serum von *S deversoir* + *S dugbe*, da im *Kauffmann-White-Schema* auf die O-Untergruppen keine Rucksicht genommen ist

Innerhalb der O 47-Gruppe gehoren zur $47_1$, $47_2$-Untergruppe *S bergen* und *S bere*, wahrend zur $47_1$, $47_3$-Untergruppe (*S kaolack*) 11 *species* gehoren Mit Hilfe von 2 Faktor-Seren $47_2$ und $47_3$ konnten alle Kulturen ohne Schwierigkeiten *in eine der O-Untergruppen eingefugt werden* Zur Diagnose dieser Kulturen in der Praxis wird ein polyvalentes O Serum von *S bergen* + *S kaolack* benutzt

Es sei aber betont, dass in speziellen, epidemiologisch wichtigen Fällen *die Differential-Diagnose* zwischen den beiden *O-Untergruppen* dieser 4 O-Gruppen gestellt werden sollte Es kommen namlich Kulturen vor, die zwar dasselbe H-Antigen besitzen, aber zu 2 verschiedenen *O-Untergruppen gehoren So besitzt zum Beispiel der originale S bergen* Stamm die Antigenstruktur $47_1$, $47_2$ : e,n,$z_{15}$, wahrend eine spater gefundene „*S bergen*"-Kultur die Formel $47_1$, $47_3$ : e,n,$z_{15}$ hat, also in Wirklichkeit eine neue *species* darstellt Obwohl wir also im allgemeinen eine Vereinfachung der serologischen Diagnose, unter Fortlassung der O-Untergruppen, empfehlen, so wollen wir trotzdem die Moglichkeit einer weiteren Differenzierung nicht aus dem Auge verlieren und in speziellen Fallen eine genaue Bestimmung der O-Untergruppen vornehmen

Zur O-Gruppe 28 gehoren bisher 42 *species*, zur O-Gruppe 40 gehoren 23 *species*, zur O-Gruppe 45 gehoren 11 *species* und zur O-Gruppe 47 gehoren 13 *species*


ZUSAMMENFASSUNG

Auf Grund einer naheren Untersuchung aller bisher bekannten 89 *species* der *Salmonella* O-Gruppen 28, 40, 45 und 47 wird uber die Herstellung *von Faktor-Seren zur Diagnose der O-Untergruppen* sowie uber die Anwendung von polyvalenten O Seren in der Routine-Diagnose berichtet Im *Kauffmann-White Schema* bleiben die O-Untergruppen unberucksichtigt, sie sollen nur in besonderen, epidemiologisch wichtigen *Fallen diagnostiziert werden*


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# BRIEF REPORT

## ANTI-Gm MOLECULES WITH DISTINCTLY DIFFERENT PHYSICOCHEMICAL PROPERTIES

By *L. Mårtensson*

Differences in the serological properties of two anti-Gm (b) sera* were observed (Mårtensson, to be published). They were therefore selected for investigation by DEAE cellulose chromatography and by density gradient ultracentrifugation. Both these sera were negative in the latex and the Waaler-Rose tests.

*On DEAE cellulose chromatography* (using increasing concentrations of NaCl buffered to pH 7.2 by Sorensen buffer as eluent) the anti-Gm of serum no. 1 was eluted *in the first peak of the chromatogram*. This peak contains the bulk of the 7 S $\gamma$-globulin. *The isoagglutinins* of the same serum were eluted in later fractions.

The anti-Gm of serum no. 2 was eluted towards the end of the chromatogram together with *inter alia* the greater part of the isoagglutinins.

The same *two sera were also subjected to density gradient ultracentrifugation* through a sucrose gradient. The anti-Gm (b) of serum no. 1 was found in fractions from the middle of the tube where the Gm (a) activity was also localized. The Gm (a) factor is *associated with 7 S $\gamma$-globulin but not with the 19 S $\gamma_1M(\beta_{2}M)$ or the* $\gamma_1A(\beta_{2}A)$ globulins (see *e.g. Grubb* 1959, *Mårtensson* 1961 a and b, *Fahey & Lawler* 1961, *Harboe et al.* 1962, *Franklin et al.* 1962). The anti-Gm (b) of serum no. 2 was found close to the bottom. The Gm (a) activity occurred in middle fractions well separated from the anti-Gm (b).

In these experiments the anti-Gm (b) of serum no. 1 behaved like the bulk of the 7 S $\gamma$-globulin, while that of serum no. 2 behaved as would be expected from a 19 S $\gamma_1M$ globulin.

An investigation of a larger number of anti-Gm sera from both healthy and rheumatoid arthritic subjects by DEAE cellulose chromatography and by density gradient ultracentrifugation is in progress and will be published together with a study of some serological characteristics of the same sera.


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* For bibliographical information for the Gm system see e.g. the reviews by *Grubb* 1961 and Ropartz 1960 and Steinberg 1962.


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# INFECTIOUS MONONUCLEOSIS WITH MULTIPLE ORGAN INVOLVEMENT COMPLICATED BY PSEUDOMEMBRANOUS LARYNGOTRACHEITIS

*An Autopsy Report*

*By*

JACOB B NATVIG

Received 1 vi 62

Infectious mononucleosis is a benign disease characterized by certain clinical, haematological and serological findings (1) While no single finding is diagnostic a positive Paul-Bunnel test (heterophile antibody determination) with absorption is regarded as the most certain evidence of infection (10), although sero-negative cases have been reported (1)

The incidence of the disease appears to be increasing (26), and it still presents many problems Although Listerella monocytogenes has been isolated from patients with infectious mononucleosis it has not been proved to be the aetiological agent (3) The disease is probably of virus aetiology, and the virus may belong to the myxovirus group *De Meio & Walker* (5) have reported that these patients show a rising titre of antibody to both the Sandai- and Newcastle disease virus It has been suggested that transmission is by "intimate oral contact", and that the infecting agent enters through the mucosa of the rhinopharynx (21)

Like the antibodies found in virus diseases the heterophile antibodies present in this condition are S 19 gammaglobulins (16), and they also have other properties in common with viral antibodies (15, 24) The relative or absolute lymphocytosis also suggests that infectious mononucleosis is a virus disease However, nobody has succeeded in isolating the causative organism or in transmitting the disease to animals or humans (4) The most recent epidemiological studies are those of *Evans* (9) *Hobson et al* (13), and *Lepine* (21)

Few patients die of infectious mononucleosis *Bennike* (1) collected 33 cases, 28 of which had been autopsied However, only 15 of these cases had been fully examined both macro- and microscopically and 3 of these [illegible]
autopsy

reported in recent years (2, 8, 18, 22, 26, 27) Six of these were autopsied, of which 4 were serologically verified Due to the scarcity of autopsy reports the present case may be of interest

## CASE HISTORY

A 17 year-old girl of healthy family and without previous serious illness was admitted to hospital on Feb 19th 1962 The tentative diagnosis was infectious mononucleosis About ten days earlier she had developed a sore throat with local glandular swelling and fever and her tonsils were covered with grey necrotic material On admission there was generalized glandular swelling The blood showed an absolute lymphocytosis, and some 'atypical lymphocytes' were present Anaemia developed and on Feb 26th sudden cardiac and respiratory arrest occurred The heart beats returned following extrathoracic stimulation and immediately afterwards tracheostomy was performed She died 16 hours later

*Serology* Paul Bunnel test Positive titre 1/448 After absorption with horse kidney titre 1/448 After absorption with ox erythrocytes titre 1/7 Wasserman and Meinicke reactions Negative

*Bacteriology* Staphylococci were cultured from the fauces

*Clinical diagnosis* Infectious mononucleosis (acute leukaemia? chloroma?)

*Post mortem examination* The autopsy was performed at the Gade Institute on Feb 27th 1962 9 hours post mortem There were some small blue petecchiae in the skin of the trunk and extremities The tonsils were large and covered by membranes Thick membranes covered the laryngeal mucosa from the epiglottis to below the level of the tracheostomy The lymph nodes in general were soft and enlarged The spleen weighed 700 g It was extremely soft and congested with blood The liver was also greatly enlarged (2880 g) The lungs showed signs of bronchopneumonia There were no macroscopical alterations in the heart gastrointestinal tract pancreas kidneys thyreoid adrenal glands bone marrow meninges or brain The serum was of a greenish colour

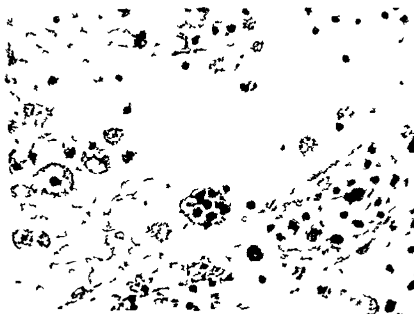
*Histology* There was a marked laryngotracheitis with membranes of fibrin mononuclear- and polymorphonuclear leucocytes and many coccoidal bacteria The underlying laryngeal wall was oedematous and infiltrated by leucocytes (Fig 1) The lymph nodes lacked their usual follicular structure and the lymphoid tissue was hyperplastic The sinuses were very wide and contained numerous large cells which showed leuco and erythrophagocytosis (Fig 2) Many of the lymphocytes were of a large atypical kind in which the nucleus was coarsely granulated and excentric and the cytoplasm vacuolated (Fig 3) The splenic capsule was thin and infiltrated by mononuclear cells There was striking disorganisation of the normal splenic structure the sinusoides were very dilated and there was some interstitial bleeding (Fig 4) The cells were similar to those in the lymph nodes There were large collections of mononuclear cells around the small bile ducts in the liver (Fig 5) The sinusoids were wide but no degenerative changes were noted in the liver cells and no fibrosis was manifest The erythro and myelopoiesis in the bone marrow was almost normal and normal numbers of megakaryocytes were present However there was a slight excess of eosinophilic cells In the heart adrenal glands and kidneys there was slight interstitial and perivascular mononuclear cell infiltration There were no degenerative changes in the cardiac muscle Coarse eosinophilic material was seen occasionally in Bowman's capsule The lungs showed alveolar leucocytic infiltration in addition ration Some of the small arteries e surrounded by mononuclear cells of tigroid substance

## DISCUSSION

The patient had a severe anaemia This is very common in leukaemia but rare in infectious mononucleosis The fulminating course also pointed to a malignant disease The heterophile antibody t

↑
*Fig 1*

La nx A ute laryng t s th memb ane to the r ght) Rema ns of ep thel al l n ng at I Haematox l n and eos n × 35



*F g 2*

pleen Wile s nu o des h ome lymphoc tes and large cell show ng leuc phago cyto s Haematoxyl n and eos n × 560)

*Fig 3*

Laryngeal wall Some 'atypical' lymphocytes (Downey-McKinlay cells) The same cell type was found in all the organs involved (Haematoxylin and eosin × 1400)



*Fig 4*

Spleen Widely distended sinusoids the remains of bleeding (Haematoxylin and eos

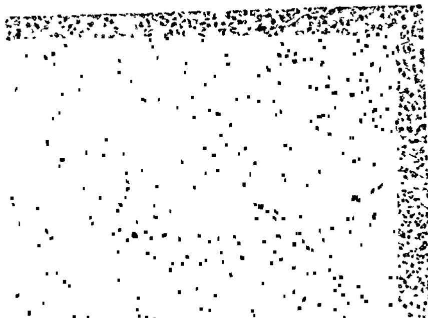


*Fig 5*

Liver Dense infiltration of mononuclear cells surrounding the bile ducts No degenerative changes in the hepatic cells (Haematoxylin and eosin × 140)



*Fig 6*

Cerebellum Arterial thickening with perivascular infiltration of mononuclear cells The same type of lesion was also found in the cerebrum (Haematoxylin and eosin × 560)

*Fig 3.*

Laryngeal wall Some "atypical" lymphocytes (Downey-McKinlay cells). The same cell type was found in all the organs involved (Haematoxylin and eosin, × 1400)



*Fig 4*

Spleen Widely distended sinusoids, the remains of a Malphigian corpuscle and bleeding. (Haematoxylin and eosin, × 35).

The skin manifestations were not investigated further, petecchial haemorrhages (25) and other exanthemata (28) are known to occur in infectious mononucleosis

The serum in this patient had a curiously green colour and the serum bilirubin was elevated (total bilirubin 4 6 mg 100 ml) As 4 3 mg/100 ml, i e 93 per cent, of this belonged to the free fraction the rise was mainly in the "prehepatic" bilirubin, of haemolytic origin (31) However, the direct Coombs' test was negative and no free atypical antibodies were found in the serum The cold agglutinin titre was elevated, as it is in some virus diseases The green colour of the serum may be due to the production of biliverdin and verdohaemoglobin-fractions as a result of the haemolytic syndrome (31) There was no excess of iron (Berlin blue stain) in the spleen, lymph nodes or bone marrow This may be due to the very short duration of the haemolysis Haemolytic syndromes are rather rare in infectious mononucleosis, but such cases do exist and have been reviewed by *Houk & McFarland* (14) Their mechanism is, however, not known but *Erwin et al* (8) quote 3 theories on the subject *In patients with infectious mononucleosis the serumglutamic-oxa*lacetic-transaminase (SGOT) is elevated, probably due to damage to the hepatic cells (20) In this case the SGOT level was 370 units (normal up to 40 units) The patient's slightly elevated serum alkaline phosphatase might be due to compression of the intrahepatic bile ducts by the cellular infiltrates (11) Electrophoresis showed a low albumin fraction and a peak in the gammaglobulins Similar findings have been described in infectious mononucleosis by *Bennike* (1)

The serological findings in the case were typical of infectious mononucleosis The Paul Bunnel titre was high It did not fall after absorp-

[illegible] nonspecific positive reactions have been described *in infectious* mononucleosis (32)

CONCLUSION

The author reports a fatal case of infectious mononucleosis in which both the pathological and serological findings support the diagnos[illegible] The [illegible]

strat[illegible]

at t[illegible] [illegible]amination in the presence of a positive Paul-Bunnel test This has been demonstrated *previously by Kass & Robbins* (17) In the present case multiple organ involvement [illegible]

[illegible]laryngotracheitis and bronchopneumonia Laryngeal obstruction due to oedema spreading from the ton-

was more suggestive of the diagnosis of infectious mononucleosis. Thus the differential diagnosis between these two conditions had to be further investigated at autopsy.

In this case the bone marrow was pleomorphic and of an almost normal aspect. This is a usual finding in infectious mononucleosis, but it is incompatible with the diagnosis of acute leukaemia. The bone marrow also showed a slight excess of eosinophilic cells. Eosinophilia in infectious mononucleosis has been reviewed by *Kauffman* (19). The picture in the liver was essentially that of an acute hepatitis. Mononuclear cells were found in the periportal tracts and in the sinusoids, but the parenchyma was intact and there was no periportal fibroblastic proliferation. Periportal infiltration may be seen both in acute leukaemia and in infectious mononucleosis. However, in the present case lymphocytes predominated in the liver, spleen and lymph nodes and also in the perivascular and interstitial infiltrations in the other organs. Many of these were like the large "atypical lymphocytes" described in infectious mononucleosis by *Downey & McKinlay* (6), and recently studied under the electron microscope by *Peagle* (23). The greatly enlarged, soft lymph nodes and disorganized splenic architecture, with widely dilated sinusoids and diffuse infiltration of mononuclear cells in the pulp, trabeculae and capsule, are typical of the findings in infectious mononucleosis (7, 29). In addition large histiocytes of the type found in the lymph nodes and spleen in the present case have been described previously in patients with infectious mononucleosis, (18).

*TABLE 1*
*Laboratory Findings*

| | Tests | Results |
|---|---|---|
| 1 | Serum bilirubin | 4.6 mg/100 ml |
| | Free fraction | 4.3 mg/100 ml |
| | Conjugated fraction | 0.3 mg/100 ml |
| 2 | Serum alkaline phosphatase | 4.5 (Bodansky units) |
| 3 | Serumglutamic oxalacetic transaminase | 370 units |
| 4 | Serumproteins | 5.8 g/100 ml |
| | Albumins | 3.7 g/100 ml |
| | Gamma globulins | 2.1 g/100 ml |
| 5 | Direct Coomb's test | negative |
| 6 | Indirect Coomb's test and papain treated cells | negative |
| 7 | Cold agglutination | titre at 4° C 128<br>titre at 18° C 2 |

The meningoencephalitis seen in this case is well known in infectious mononucleosis (2) but very rare in acute leukaemia.

The infiltrates found in the heart, kidneys, lungs, and adrenal glands are also compatible with the diagnosis of infectious mononucleosis (8, 12).

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sils into the larynx is well known in infectious mononucleosis (29, 30), and pseudomembranous pharyngitis is not rare (18, 28) However, there is no previous record, in the available literature, of pseudomembranous laryngotracheitis causing death in infectious mononucleosis

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# RENAL HYPERTENSION IN RENIN DEPLETED RATS

*(Relation between Renal Renin Content and Hypertension II)*

by

JENS BING

Received 27 xi 62

Overdosage with sodium retaining corticoids and salt is known to produce increased blood pressure and decreased renal renin content in dogs and rats (*Bessinger & Wakerlin 1948, Sevy & Wakerlin 1953, Gross & Sulser 1956, Gross* 1960) In most cases the increase in blood pressure is slight compared with the more marked hypertension in animals with partial constriction of the renal arteries It is therefore of interest to investigate whether renal hypertension can develop in desoxycorticosterone and salt treated animals with renin depleted kidneys Apparently such studies have been performed only by *Sevy & Wakerlin* (1953), who found development of pronounced hypertension after renal artery constriction in three desoxycorticosterone acetate treated dogs in which the renal renin concentration was reduced They concluded that this could be interpreted as evidence against the renin hypothesis

The object of the following experiments was primarily to determine if bilateral partial constriction of the renal arteries produces the same degree of hypertension in renin depleted as in normal rats, and secondarily to study the effect of the clamping on the renal renin content in desoxycorticosterone and salt treated rats In order to determine the significance of the relation between renal renin content and hypertension studies on the correspondence between renal renin content and renin release were required As the renin release cannot be determined directly an estimation of the release was attempted by comparing the intensity of the blood pressor response to renin in unilaterally clamped rats after removal of either the clamped renin-rich or the untouched renin-poor kidney the results being compared with the response of both normal, non-nephrectomized unilaterally clamped and bilaterally nephrectomized rats

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## MATERIAL AND METHODS

The material included 66 albino rats of which 18 were untreated controls 10 had bilateral partial constriction of the renal arteries 24 were treated by subcutaneous injection of 5 mg microcrystalline Percortene (Ciba) for about 4 months and thereafter 2 5 mg per 100 g body weight twice a week and 14 were both treated with Percortene and operated with uni or bilateral clamping of the renal arteries While the first two groups (which were included in a previous publication (*Bing* 1962)) had water the last two groups received 0 9 per cent saline solution as drinking fluid The hormone and salt treatment was started when the rats were about 1 1½ months old In addition a further 34 female albino rats were employed to study the responsiveness to intravenously injected renin

Hypertension was induced by the method of *Byrom & Wilson* (1938) Blood pressure determinations were made with the plethysmographic apparatus described by *William Harrison & Grollmann* (1939) either without anaesthesia or during recovery from a light ether anaesthesia

The kidneys were frozen and thawed three times as described by *Haas Lamfrom & Goldblatt* (1954) The extracts were prepared in glass homogenizers after dilution of the minced tissue with either 4 or 9 volumes of 0 9 per cent sodium chloride After homogenization the extracts were stored for about 18 hours at 4° C with a small amount of toluene and centrifuged The supernatant was generally diluted further before being used for the assay

The *renin assay* was performed by determination of the pressor response to intravenous injection of the extracts in anaesthetized female rats weighing 160–180 g using the method of Skeggs Kahn & Marsh (1953) The test rats were pretreated with 0 005 mg ergotamine tartrate The pressor response was compared with that of a standard renin preparation each extract being tested at least in duplicate 0 05 ml of the standard renin diluted 1 to 100 with 0 9 per cent sodium chloride, was taken as equivalent to 1 unit of renin activity The renal renin concentration was said to be 100 per cent of the normal if 0 05 ml of 1 in 120 dilution of the homogenate gave the same pressor response as 0 05 ml of the 1 per cent standard renin solution To this dose most of the test rats responded with increase in blood pressure of about 12 mm

## RESULTS

### 1 *Controls*

The 18 untreated controls, kept in our animal house for about the same time as the hormone treated rats, had a *blood pressure* usually in the range 80 and 140 with higher values (maximally 180 mm) in a few instances The relative heart weight (in per cent of body weight) was in all cases within the normal limits from 0 25 to 0 33 per cent The *renal renin concentration* was about 60 per cent (dispersion 30 to 125 per cent) of the "normal renin content" of rats killed immediately on receipt from the dealer (Fig 1 A) The decrease in renin content may be an indication of a hormonally conditioned stress reaction to the handling of the rats The *total amounts of renin* in one kidney was found to be between 750 and 2500 units (Fig 2 A)

### 2 *Effects of Bilateral Clamping*

*Blood pressure* 6 of the 10 bilaterally clamped rats were hypertensive (Fig 3 A) with increased relative heart weight in the range 0 32 to 0 48 per cent In the other 4 rats with bilateral partial constriction of the

I express my thanks to *Ciba Ferrosan and Leo Pharmaceutical* for having supplied me with Percortene Renin and Heparin respectively

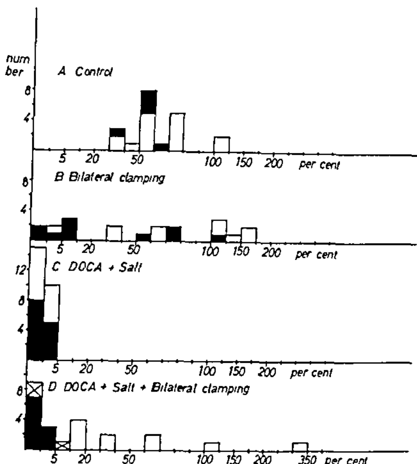



*Fig 1*

☐ lighter and ■ heavier of the two kidneys
☒ unilaterally clamped kidney in previously unilaterally nephrectomized rat
The abscissa gives the renal *renin concentration* in per cent of the average value found in normal rats killed upon arrival at the institute The changes of scale should be noted The ordinates give the number of rats in the different groups The control rats (A) which were kept at the institute for the same time as the following groups had about half the 'normal' content Bilateral clamping most often resulted in a decreased renin concentration in the heavier and unchanged or slightly increased value in the lighter of the two kidneys (B) Treatment with desoxycorticosterone acetate and salt produced the well known pronounced decrease in renin concentration (C) Bilaterally clamped DOCA plus salt treated rats showed the same extremely low renin concentration in the heavier and most often a subnormal to normal concentration in the lighter of the kidneys In unilaterally nephrectomized rats belonging to this group the renin concentration was extremely low (D)

renal arteries the blood pressure remained normal (Fig 6), but three of these animals had increased heart weight (from 0 39 to 0 48 per cent) and hypertensive vascular disease, the fourth showed loss in weight, a relative heart weight of 0 32 per cent, pareses and hypertensive vascular disease and died 12 days after the operation, so that only a few blood pressure determinations could be performed The *renin concentration* was normal or slightly increased in one kidney and normal or decreased in the other, the higher concentration occurring usually in the lighter kidney (Fig 1 B) The *ratio* between the concentrations

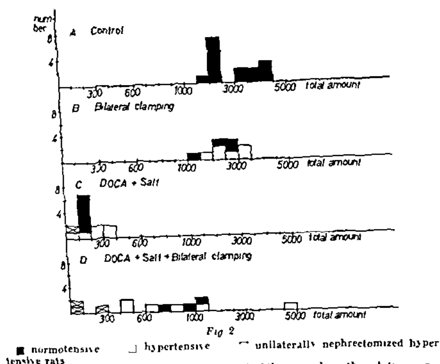



Fig 2

■ normotensive □ hypertensive ⌐ unilaterally nephrectomized hypertensive rats

in the two kidneys which is normally in the range 1 to 1 5 had increased (from 1 6 to 88) in all but one case These changes were found in both normo- and hypertensive animals The *total amount of renin measured* per kidney varied between 1104 to 3400 units in the kidney with the higher renin content, and between an undetectable level *and 1560 units* in the kidney with the lower renin content, whilst the total renin content in both kidneys was in the range 1300 to 3400 units (Fig 2 B)

### 3 Effects of Desoxycorticosterone and Salt Treatment

In 38 rats which had been pretreated for $2\frac{1}{2}$ months and were continuously treated with desoxycorticasterone and salt, the *blood pressure* was followed every second day *for about 3* weeks After this period of time 13 normotensive were chosen for the operation of partial constriction of the renal arteries (Fig 3 B). The hormone and salt treatment of the remaining 25 rats was continued for up to about 7 months

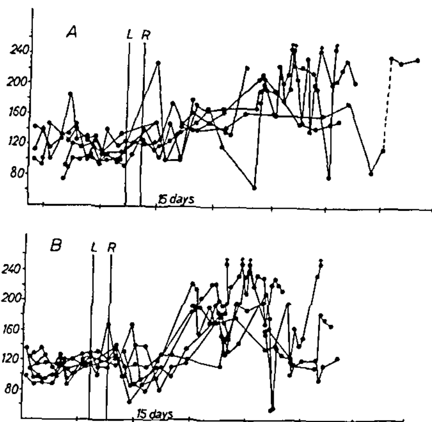



Fig 3

*Blood pressure before and after clamping of left (L) and right (R) renal artery in normal (A) and desoxycorticosterone acetate and salt treated rats (B)* The treatment of the animals in groups B was started about 3 months before and continued after the clamping The points indicated with an arrow mark the upper limit 250 mm Hg, which could be measured with the method used

It is clearly seen that the animals of both groups respond by exhibiting renal hypertension and that the blood pressure curves are significantly different from those found in DOCA plus salt treated rats with untouched renal arteries (fig 4 A and B)

Of these 25 rats 6 were completely or nearly normotensive, 7 had a slight (Fig 4 A) and 12 a moderate hypertension (Fig 4 B) The blood pressures of these two groups of 13 and 12 rats are given in two figures (Fig 4 A and B) in order to show that the animals which had a normal or slightly elevated blood pressure after 2½ to 3 months, continued to have such rather low values Similarly the animals which started with higher blood pressures in most cases continued to exhibit blood pressure in the same range It should be noted that the values are significantly lower than the pronounced hypertensive values found in the renal hypertensive rats mentioned previously (Fig 3 A)

The *relative heart weight* was found to be normal or slightly increased, the average value was 0 39 (range 0 35 to 0 43) per cent in the group with the lower blood pressure (Fig 4 A) and slightly higher, about 0 42 (range 0 37 to 0 53) per cent, in the group with the higher blood pressure (Fig 4 B) The only exceptions were two anaemic ani-

mals with heart weights of 0 47 and 0 64 and haemoglobin percentages of 68 and 76 per cent, respectively

As some of the animals died spontaneously determinations of the renal *renin concentration* could be performed in only 18 of the hormone and salt treated rats, which were killed from 1½ to 8½ months after the start of the treatment In all of these 18 animals the renin concentration was found to be extremely low Most of the results obtained ranged between 2 and 4 per cent of the normal value, while in two single instances values in the ranges 4 to 5 and 5 to 6 per cent were recorded (Fig 1 C) The concentrations in right and left kidneys were usually identical, giving a ratio of 1 0, though in two cases a ratio of 2 0 was found In 6 kidneys from 5 of the 18 rats the renin concentration was so low that it was undetectable by our method, which means that these contained less than 2 per cent of the "normal" content No connection was found between the duration of hormone treatment and the percentage amount of renin in the kidneys

The *weight of the kidneys* had in all cases significantly increased above the normal values, which was noted both by direct determination and by calculation of the relative weight Normally the weight of one kidney in animals of these sizes amounts to 0 25 to 0 40 per cent of the body weight, but in the hormone treated animals the values represented from 0 45 to nearly 0 8 per cent, the highest weight for a single kidney was 2 4 g, found in a rat weighing well over 300 g Although the increase in renal weight is pronounced, it is small as compared with the marked decrease in renin concentration The values for the *total amount of renin per kidney* are low, ranging from undetectable amounts to a maximum of 190 units in one kidney, compared with 750 to 2500 units in single kidneys from control rats The *total renin content of both kidneys* was in all cases less than 400 units (Fig 2 C)

In four rats which were unilaterally nephrectomized it was found that *cessation of the hormone and salt treatment* for four, eight, twelve, and eighteen days did not influence either the high weight, or the low renin content of the remaining kidney

4 *Effects of Partial Constriction of one or both Renal Arteries in Renin-Depleted Rats*

The effects of partial constriction of one or both renal arteries were studied in 14 rats 10 of which had both renal arteries constricted, the remaining four having had the left renal artery constricted and the right kidney removed As mentioned above these rats were chosen from the hormone and salt treated rats which were normotensive after 3 (13 cases) to 7 months treatment In 10 of the animals the operation resulted in a significant increase in the *blood pressure* with curves (Fig 3 B) similar to those seen after the same operation on normal rats (Fig 3 A) and different from those obtained in non-operated hormone

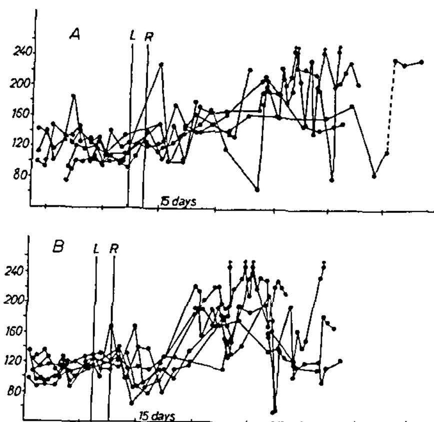



*Fig 3*

*Blood pressure before and after clamping of left (L) and right (R) renal artery in normal (A) and desoxycorticosterone acetate and salt treated rats (B)* The treatment of the animals in groups B was started about 3 months before and continued after the clamping The points indicated with an arrow mark the upper limit 250 mm Hg, which could be measured with the method used

It is clearly seen that the animals of both groups respond by exhibiting renal hypertension and that the blood pressure curves are significantly different from those found in DOCA plus salt treated rats with untouched renal arteries (fig 4 A and B)

Of these 25 rats 6 were completely or nearly normotensive, 7 had a slight (Fig 4 A) and 12 a moderate hypertension (Fig 4 B) The blood pressures of these two groups of 13 and 12 rats are given in two figures (Fig 4 A and B) in order to show that the animals which had a normal or slightly elevated blood pressure after 2½ to 3 months continued to have such rather low values Similarly the animals which started with higher blood pressures in most cases continued to exhibit blood pressure in the same range It should be noted that the values are significantly lower than the pronounced hypertensive values found in the renal hypertensive rats mentioned previously (Fig 3 A)

The *relative heart weight* was found to be normal or slightly increased, the average value was 0 39 (range 0 35 to 0 43) per cent in the group with the lower blood pressure (Fig 4 A) and slightly higher, about 0 42 (range 0 37 to 0 53) per cent, in the group with the higher blood pressure (Fig 4 B) The only exceptions were two anaemic ani-

increased relative heart weight of 0 56 per cent and hypertensive vascular disease

The *renin concentration* was determined in 13 of the 14 hormone and salt treated rats which had one or both renal arteries constricted 10 of these animals had bilateral renal artery constriction In these the renin concentration in the heavier of the two kidneys (Fig 1 D) was in the same low range as were the values obtained from non operated, hormone and salt treated rats (Fig 1 C), most of them being below the threshold of the method The renin concentration in the lighter of the two kidneys (Fig 1 D) was distinctly higher than the values in corresponding kidneys from the non operated hormone treated rats (Fig 1 C) and was on an average lower than those found after renal artery constriction in otherwise untreated rats (Fig 1 B) The *ratio* between the concentrations in the two kidneys was abnormally high from 3 5 to 214 In the remaining 4 rats the right kidney was removed three days after clamping and in no case did it contain detectable amounts of renin These measurements are not included in Fig 1 D, which shows only the values obtained from 3 of the 4 remaining kidneys of the unilaterally nephrectomized rats, as the fourth animal died spontaneously and no autopsy was performed The renin concentrations of these 3 kidneys, which were removed 1½ to 2 months after the artery constriction were in all cases low, being "below threshold", 1 4 and 7 per cent, respectively In all four cases the *relative weight of the right kidneys*, which were removed, were high values characteristic of desoxycorticosterone and salt treated rats, namely 0 43, 0 47, 0 53 and 0 59 per cent of the body weight It is of interest that although the renal arteries were partially clamped the unilateral nephrectomy produced a further "compensatory" hypertrophy of the remaining kidneys, resulting in such high relative weights as 0 74, 1 2 and 1 2 per cent, with corresponding increases in the kidney weights in grams since there was little alteration in the body weights

The results of determinations of the *total renin content* in the two kidneys of the ten bilaterally operated rats and in the remaining kidneys in three of the four unilaterally nephrectomized rats are given in Fig 2 D It is seen that while most animals, including the three with unilateral nephrectomy, had significantly decreased values, a few had

creased value The figure

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5 *Relation between Renal Renin Content and Responsiveness to intravenously Injected Renin*

The degree of response to a standard dose of renin was studied in the many normal rats, which served as test animals in the above mentioned

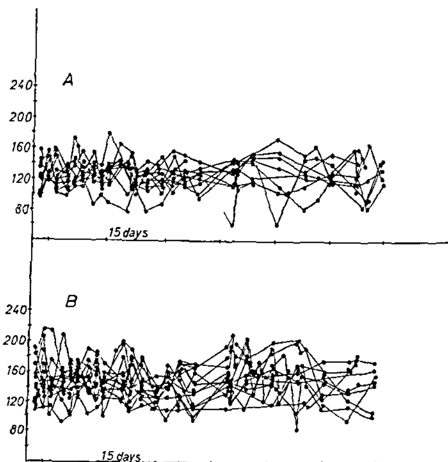



Fig 4

*Blood pressure of two groups of desoxycorticosterone acetate and salt treated rats* the curves starting about 3 months after the treatment was commenced The first group (A) presented normal or slightly increased and the second (B) significantly increased values The fact that in both groups the values are fairly constant during many months shows that the pronounced increase in blood pressure following clamping of the renal arteries in normotensive DOCA plus salt treated rats (fig 3 B) is a real renal hypertension

and salt treated rats (fig 2 A and 2 B) In accordance with this the relative *heart weight* was higher, averaging 0 54 (range 0 51 to 0 67) per cent, when a single case of an anaemic rat with a value of relative heart weight of 0 82 and a haemoglobin per cent of 82, was excepted The remaining 4 rats were all normotensive (Fig 6) One of these died spontaneously only a week after the operation Of the other three one was killed about 3 weeks after the operation because of debility, pareses, and twitchings of the head Autopsy showed an increased heart weight of 0 62 per cent, pancreatic oedema, and hypertensive vascular disease The second which was killed about 1½ months after the operation, was anaemic with a haemoglobin per cent of 80, autopsy showed pancreatic oedema, a high relative heart weight of 0 65 per cent, and hypertensive vascular disease The last normotensive rat was debile and died spontaneously about 2 weeks after the operation, autopsy showed an

increased relative heart weight of 0 56 per cent and hypertensive vascular disease

The *renin concentration* was determined in 13 of the 14 hormone and salt treated rats which had one or both renal arteries constricted 10 of these animals had bilateral renal artery constriction In these the renin concentration in the heavier of the two kidneys (Fig 1 D) was in the same low range as were the values obtained from non operated, hormone and salt treated rats (Fig 1 C), most of them being below the threshold of the method The renin concentration in the lighter of the two kidneys (Fig 1 D) was distinctly higher than the values in corresponding kidneys from the non operated hormone treated rats (Fig 1 C) and was on an average lower than those found after renal artery constriction in otherwise untreated rats (Fig 1 B) The *ratio* between the concentrations in the two kidneys was abnormally high – from 3 5 to 214 In the remaining 4 rats the right kidney was removed three days after clamping and in no case did it contain detectable amounts of renin These measurements are not included in Fig 1 D, which shows only the values obtained from 3 of the 4 remaining kidneys of the unilaterally nephrectomized rats, as the fourth animal died spontaneously and no autopsy was performed The renin concentrations of these 3 kidneys, which were removed 1½ to 2 months after the artery constriction were in all cases low, being "below threshold , 1 4 and 7 per cent, respectively In all four cases the *relative weight of the right kidneys,* which were removed, were high values characteristic of desoxycorticosterone and salt treated rats, namely 0 43, 0 47, 0 53 and 0 59 per cent of the body weight It is of interest that although the renal arteries were partially clamped the unilateral nephrectomy produced a further "compensatory" hypertrophy of the remaining kidneys, resulting in such high relative weights as 0 74, 1 2 and 1 2 per cent, with corresponding increases in the kidney weights in grams since there was little alteration in the body weights

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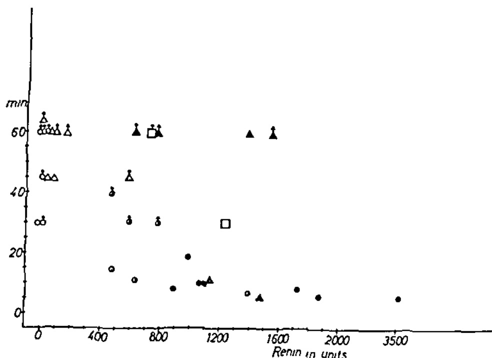



*Fig 5*

*Normal rats* ● nonoperated, ◑ unilaterally nephrectomized ○ bilaterally nephrectomized
*Unilaterally clamped rats* □ nonoperated ▲ after removal of the untouched kidney △ after removal of the clamped kidney
The figure shows the *relation between the total renal renin content and the responsiveness to intravenously injected renin* as measured by the duration in minutes from the injection to the return of the blood pressure to its starting value Sign marked with an arrow indicates that the starting value had not been reached at the time shown

There is an inverse correlation between the two values in all groups except the group having clamped kidneys Here the correlation did not hold in the majority of the rats

determinations of the renal renin content From this it is evident that the blood pressure in most normal rats returns to its starting point 5 to 15 minutes after the intravenous injection of renin When the same dose was injected into rats, which were nephrectomized 6 to 24 hours before the test, the response was higher and more prolonged as seen in Fig 5, in which the duration of the response is plotted against the total renin content of the test animals (These determinations were *performed with a standard renin preparation which was different from* the standard used in the previous parts of this work) Similar studies performed on unilaterally clamped rats after removal of the clamped kidneys showed *that these rats, having a very low renin content reacted* with a similarly long response The figure also shows that unilaterally nephrectomized normal rats, which have about half the normal renin content have a response *duration intermediate between those of normal* and nephrectomized rats So far there is an inverse proportionality between the renal renin content and the response to intravenously in-

jected renin. Studies on unilaterally clamped rats which were either non nephrectomized or had the renin poor untouched kidney removed, however showed that these in most cases had a longer response time than non nephrectomized or unilaterally nephrectomized nonclamped rats with similar renal renin content.

## DISCUSSION

### 1 *Effect of Bilateral Clamping on Renal Renin Content in Desoxycorticosterone Acetate and Salt Treated Rats*

In accordance with the previously mentioned studies treatment with desoxycorticosterone acetate and salt was found to result in a pronounced decrease in both concentration and total amount of renin, the values being in nearly all cases reduced to less than 10 per cent, and in many cases below the threshold of determination with our method (Fig 1 C and 2 C). Clamping of the renal arteries of hormone treated animals resulted in the same marked decrease in the renin concentration of the heavier kidney as *Gross & Lichtlen* (1958) found in the untouched kidney in unilaterally clamped rats. In the lighter kidney, however, the increase in pressor material found by *Gross & Lichten* in the clamped kidneys of DOCA pretreated rats was found in only a few cases, most of these kidneys having a lower renin content (Fig 1 D) than the corresponding kidneys from bilaterally clamped rats which did not receive hormone and salt (Fig 1 B). This difference resulted in significantly lower values for the total amount of renin in the hormone treated group (Fig 2 D) compared with the other animals (Fig 2 B). It is thus reasonable to believe that the extremely low values in the heavier and the moderately decreased values in the lighter kidneys result from a summation of 1) the decrease found in both kidneys in hormone treated rats (Fig 1 C) and 2) the tendency for a decrease in the heavier and an increase in the lighter of the two kidneys found in bilaterally clamped rats (Fig 1 B). This view is supported by the very low renin content which is found in the remaining clamped kidneys of hormone and salt treated rats after unilateral nephrectomy (Fig 1 D and 2 D), the decreased values being in accordance with recent studies by *Regoli et al* (1962). These workers showed that the increased renin concentration in the unilaterally clamped kidney *returns to normal* after removal of the untouched kidney. Quite similarly the subnormal values of our both clamped and hormone plus salt treated rats decreases to extremely low values after unilateral nephrectomy.

### 2 *Relation between Renal Renin Content and Hypertension*

In the previous paper on the relation between renal renin content and hypertension (*Bing* 1962) it was shown that in the first 3½ months after partial constriction of one or both renal arteries the total content

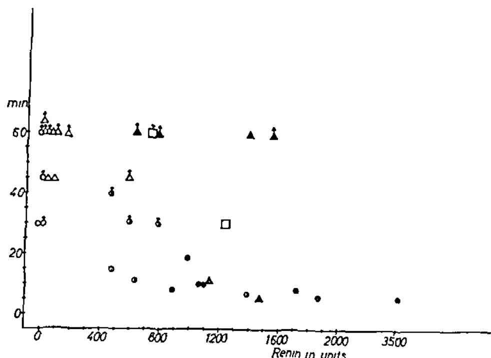



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plus salt treated rats Furthermore *Omae, Masson & Page* (1960 and 1961) have shown that while grafting of normal and clamped kidneys onto 24 hour nephrectomized recipients results in a renin like pressor-response, no pressor activity is demonstrated when kidneys from DOCA and salt treated rats are grafted *Gross* (1958) mentions some (un published?) experiments showing a normal responsiveness in uni laterally clamped rats after removal of the untouched kidneys, and as the clamped kidneys have increased renin content this finding is in agreement with all of the results mentioned above, making it *probable both that the response to intravenously injected renin depends on the renin release, and that the renin release follows the total renin content* The present experiments however, show that most of the unilaterally clamped rats possessing either both or only the clamped kidney reacted with a prolonged pressor response This result cannot be explained in accordance with the above conclusions The reason for this exception to what seems to be the general rule could be either that the renin release is decreased in clamped kidneys or that some unknown factor prolongs the duration of the renin response in rats with clamped kid neys In the present study on the development of renal hypertension in renin depleted rats it is of interest that rats with renin depleted kidneys respond in the same way as bilaterally nephrectomized rats Both the lack of correlation shown previously between the degree of blood pressure elevation and the total sum of renin in the two kidneys and the present demonstration of the development of the same degree of [illegible]
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*Bon & Hoobler* (1960) in which it was shown, that isovolemic cross circulation between a renal hypertensive and a normal rat did not result in any increased pressure in the normal rat

### 4 *Hypertensive Disease without Demonstrable Hypertension*

8 of the rats with renal artery constriction remained normotensive (Fig 6) Autopsies performed in 7 of these showed signs of hypertensive cardiovascular disease namely pancreatic oedema hypertensive vascular disease, and hypertrophy of the heart In accordance with this two of these normotensive rats had clinical signs of hypertensive en cephalopathy pareses, and twitchings Some of the animals lived only one to two weeks after the operation so that few blood pressure determinations were performed The normotensive values however, agree well with the normotension found in the same period after renal artery constriction in rats which later exhibited hypertension (Fig 1 A and B) [illegible]

of renin in the two kidneys is almost always normal and that in animals surviving for more than 3½ months after the constriction well over half have a total renin content exceeding the normal values No closer correlation between the degree of blood pressure elevation and the total sum of renin in the two kidneys could be demonstrated at any time after partial constriction of the renal arteries The lack of correlation between blood pressure and renal renin content has recently been demonstrated by *Regoli et al* (1962) It is still more evident in the present study in which renal hypertension is induced in desoxycorticosterone and salt treated rats having an extremely low renin content (Fig 2 C), which in most cases remains low, and in only a few instances rises to a subnormal or slightly increased (one case) value (Fig 2 D) Although these animals were treated with desoxycorticosterone and salt the hypertension was clearly of the renal type, the blood pressure curves (Fig 3 B) were different from those found in non-operated hormone and salt treated rats (Fig 4 A and B), but very similar to those found after renal artery constriction on normal rats (Fig 3 A)

3 *Relation between Renal Renin Content and Renin Release*

The fact that *renal hypertension can be produced in renin depleted rats* agrees well with the studies on 3 dogs (*Sevy & Wakerlin* 1953) mentioned previously In order to discover if renin plays any rôle in the pathogenesis of renal hypertension it is thus of the greatest importance to know if lack of demonstrable renin and very low renin content are signs of a concurrent pronounced decrease in renin release So far the renin release cannot be directly determined As an indirect estimate the responsiveness to intravenous injection of renin was studied in non-operated and bilaterally or unilaterally nephrectomized normal rats and in unilaterally clamped rats which were non-nephrectomized or had either the clamped or the untouched kidney removed In this way it was found that the length of the pressor response to renin is inversely proportional to the total renal renin content when studied in normal, unilaterally and bilaterally nephrectomized rats and in unilaterally clamped rats after removal of the clamped kidneys These results are in good agreement with previous studies by *Tigerstedt & Bergman* (1898), *Mc Cubbin & Page* (1954), *Masson et al* (1954 55), *Gross & Sulser* (1954, 1956), and the last two teams have further shown, that desoxycorticosterone acetate plus salt treated rats having renin depleted kidneys react with the same prolonged pressor curve as bilaterally nepreetomized rats From this it is reasonable to conclude that the length of the pressor effect is determined by the renin release This assumption is further supported by experiments of *Gross & Sulser* (1954) showing that in bilaterally nephrectomized rats the response is normalized by intraperitoneal injections of extracts of normal kidneys but uninfluenced by extracts of renin depleted kidneys from DOCA

plus salt treated rats Furthermore *Omae, Masson & Page* (1960 and 1961) have shown that while grafting of normal and clamped kidneys onto 24 hour nephrectomized recipients results in a renin like pressor-response, no pressor activity is demonstrated when kidneys from DOCA and salt treated rats are grafted *Gross* (1958) mentions some (unpublished?) experiments showing a normal responsiveness in unilaterally clamped rats after removal of the untouched kidneys, and as the clamped kidneys have increased renin content this finding is in agreement with all of the results mentioned above, making it *probable both that the response to intravenously injected renin depends on the renin release, and that the renin release follows the total renin content* The present experiments, however, show that most of the unilaterally clamped rats possessing either both or only the clamped kidney reacted with a prolonged pressor response This result cannot be explained in accordance with the above conclusions The reason for this exception to what seems to be the general rule could be either that the renin release is decreased in clamped kidneys or that some unknown factor prolongs the duration of the renin response in rats with clamped kidneys In the present study on the development of renal hypertension in renin depleted rats it is of interest, that rats with renin depleted kidneys respond in the same way as bilaterally nephrectomized rats Both the lack of correlation shown previously between the degree of blood pressure elevation and the total sum of renin in the two kidneys and the present demonstration of the development of the same degree of [illegible]

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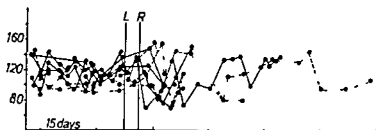



*Fig 6*

The figure gives the *blood pressure of 8 rats which developed hypertensive cardio vascular disease although they remained normotensive after clamping of left (L) and right (R) renal artery* The curves marked by unbroken lines represent the values from rats treated with DOCA and salt, and the broken lines show the values in rats which were not subjected to this treatment

vation of arterial pressure is only one of the pathogenic factors in the hypertensive vascular disease The occurrence of hypertensive vascular disease in normotensive animals can, however, also be interpreted as a sign of inadequacy of the indirect method of blood pressure determination The fact that direct blood pressure determinations in some cases, which are not included in this material, have revealed hypertensive values in rats in which the indirect method showed normotension, speaks for this interpretation, which is also supported by the increased heart weight found in most of these rats

5 *Renal Hypertrophy in Desoxycorticosterone and Salt Treated Rats*

Although desoxycorticosterone acetate is not included in the list of renotrophic factors mentioned by *Braun-Menendez* (1952) it has been shown to produce renal hypertrophy in rats by *Selye* (1950), who found distension of the convoluted tubules with some hypertrophy and hyperplasia of the lining cells The glomeruli were found to be hyperaemic and in some cases glomerular hyalinization and hyalin necrosis of the arterioles ensued In the present study the hormone treatment even where it was continued for up to 6 months resulted in hypertrophy of the kidney without glomerular damage In accordance with the histological picture blood urea was found to be normal In some of these cases unilateral nephrectomy and clamping of the renal artery of the remaining kidney was followed by further increase in renal weight Here the apparent further "hypertrophy' may be due to the following pronounced histological abnormalities dilation of many tubules with precipitates in some, and peritubular infiltration with lymphocytes, plasma cells, and considerable amounts of connective tissue

SUMMARY

(1) In desoxycorticosterone acetate and salt treated rats the well known decrease in renal renin concentration was observed, the values in many cases being below the threshold of detection of the method

(Fig 1 C and 2 C) When animals pretreated in this way had both renal arteries clamped, the renin concentration remained extremely low in the heavier of the kidneys In the lighter kidney the concentration increased (Fig 1 D), but was lower on the average than the one found in the lighter kidney on bilaterally clamped but otherwise untreated rats (Fig 1 B) In unilaterally nephrectomized hormone treated rats clamping of the remaining renal artery did not result in any increase in the renal renin content (Fig 1 D) The observed changes in renin concentration can most readily be explained as a summation of the effects of the two treatments, 1) hormone plus salt and 2) clamping of the renal arteries

(2) *Renal hypertension could be produced in renin depleted (Fig 3 B) as well as in normal rats (Fig 3 A) although the total renal renin content remained extremely low in some animals and subnormal in most cases* (Fig 2 D)

(3) Previous work and the present (Fig 5) studies on the relation between the total renal renin content and the degree of response to intravenously injected renin together make it probable, that a decrease in renal renin content is followed by decreased release of renin *The production of renal hypertension in renin depleted rats can thus be taken as one piece of evidence against the theory stating that renal hypertension is due to a hypersecretion of renin* The fact that some unilaterally clamped rats reacted with a more prolonged pressor response to renin than normal rats with about the same total renal renin content (Fig 5) also speaks against this theory

(4) Some rats which remained normotensive after bilateral clamping (Fig 6) developed hypertensive cardiovascular disease This could either be explained by the existence of some pathogenetic factor other than the high blood pressure or by an inadequacy of the indirect method for blood pressure determinations

(5) Treatment with desoxycorticosterone and salt was followed by pronounced renal hypertrophy without significant renal damage Further increase in renal weight after clamping of the renal arteries was apparently due to dilated tubules and infiltration with lymphocytic cells and connective tissue

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FROM THE UNIVERSITY DEPARTMENT OF PATHOLOGY GENERAL HOSPITAL MALMÖ SWEDEN

# STUDIES ON VASCULAR CHANGES IN CHRONIC LUNG CONGESTION

## I Blood Vessels

by

GIUSEPPE FICHERA[1] and INGA HAGERSTRAND

Received 28 vi 62

The literature on vascular changes in chronic lung congestion is voluminous Investigations have been carried out on operative and necropsy specimens (3 5 24 12 13 14 29), as well as on experimental animals (2 20) Despite this wide attention opinions differ on the interpretation of the findings In the present investigation we studied the vascular changes in the lungs in secondary hypertension of the pulmonary circulation

TABLE 1

*Survey of Material*

| Age | Cases with mitral stenosis | | | Rest of the material | Control material |
|---|---|---|---|---|---|
| | males | females | total number | | |
| 0–9 years | | | | | 4 |
| 10–19 years | | | | | 2 |
| 20–29 years | | 1 | 1 | | 2 |
| 30–39 years | 1 | 1 | 2 | 4 | 5 |
| 40–49 years | 6 | 5 | 11 | 4 | 8 |
| 50–59 years | 11 | 15 | 26 | 21 | 8 |
| 60–69 years | 6 | 25 | 31 | 16 | 8 |
| 70–79 years | 6 | 17 | 23 | 24 | 8 |
| 80–89 years | 1 | 5 | 6 | 6 | 5 |
| | 31 | 69 | 100 | 75 | 50 |

## MATERIALS AND METHODS

The material ...
ment of Pat...
pulmonar...
sufficienc...
not mitra... ...of myocardial infarcts with
chronic hea(rt failure) and of 50 controls showing no signs of chronic lungcongestion

[1] Present address Institute of Pathology University of Catania Italy



*Fig 1*

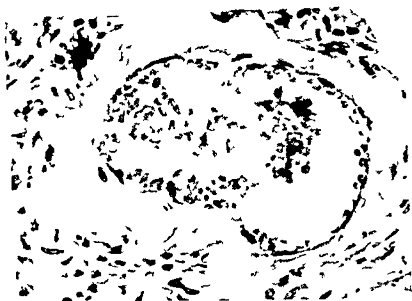
Contracted muscular artery with thickening of the media and hyperplasia and splitting of elastic fibres Weigert's elastica × 192

(see Table 1) Both lungs were examined histologically, and in most cases sections were available from each lung lobe The sections were fixed in formalin and embedded in paraffin Haematoxylin erythrosin van Gieson, Weigert's elastica and Turnbull's stain for iron were used

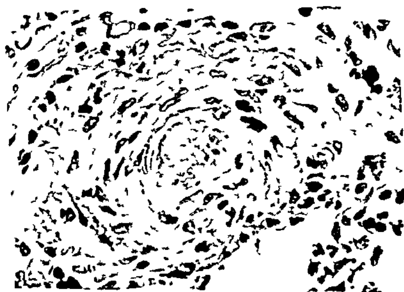
## RESULTS

In the description of the vascular changes in lung congestion no difference will be made between mitral disease and the remainder since the changes did not differ in kind but only in severity they were more advanced in the mitral disease, particularly in muscle hyperplasia of the arterioles

Large arteries in the lungs, even in young persons, showed patchy intimal thickening of arteriosclerotic type, sometimes with atrophy of the media at the site corresponding to the intimal lesion In the muscular arteries of larger and smaller calibre (between 1000 and 100 $\mu$ in diameter) intimal fibrous thickening was seen sometimes, but it was not always so marked The essential change consisted of a thickening of the media because of muscle hyperplasia and was often most impressive in the small, muscular arteries (Figs 1, 5) Fibrohyalinosis of the media was common in these vessels (Fig 2) Hyperplasia of the elastica layer was also common, sometimes with splitting of the elastic fibres (Fig 1) Signs of vascular contraction were frequently demonstrable by a palissade-like arrangement of vascular endothelium and folding of the elastica (Fig 1) Arterioles (diameter less than 100 $\mu$)



F g 2
F br h al nos s of the wall of a small artery Van G eson × 480



F g 3
Arteriole with ad anced mu ular thicken ng of the med a Haemat xyl n erythrosin × 48

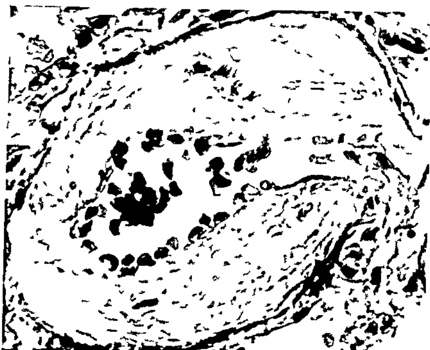


*Fig 4*
Arteriole with fibrohyalinosis of the wall Van Gieson × 700

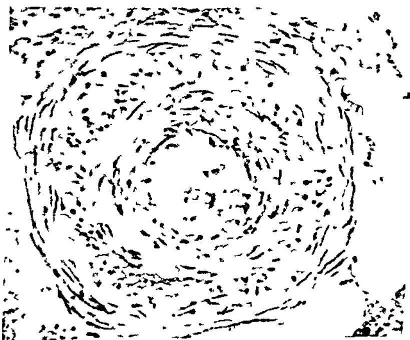


*Fig 5*
Artery with thickening of the media Haematoxylin erythrosin × 160

showed a clear thickening in the muscular layer in the media which is normally very scanty (Fig 3) The media also often showed fibrohyalinosis of varying degree (Fig 4) The adventitia usually exhibited no substantial changes Only in a few cases was any clear thickening with fibrohyalinosis seen, apart from the mural thickening of the lymphatics along the adventitia (8) Veins of varying caliber often showed intimal, fibrous thickening

As to the control material, which was grouped according to age (see Table 1), vascular changes were not regularly seen in subjects below 50 years These changes then increased with age (The physiologically thicker vascular walls in newborns also received attention) The changes consisted of intimal fibrosis and fibrohyalinosis of the media of medium sized and small arteries as well as in arterioles Also the veins showed intimal thickening with fibrosis Mainly these vascular changes seen also in the lungs of chronic stasis were less advanced in the controls

TABLE 2

*Degree of Mitral Stenosis*

| Degree of stenosis of the mitral c valve accord ng to Henry[14] | Number of cases |
|---|---|
| | - |
| Degree of stenosis not known in rest of cases | 78 |

In the analysis of our observations we grouped the cases according to age, duration of basic disease and in cases of mitral stenosis, according to the degree of stenosis (see Table 1, 2) We found no correlation between age and severity of the vascular changes Nor was any co variation found between the duration of the basic disease as judged from clinical data (duration of basic disease varied between 1–48 years in cases of mitral disease, between 1–10 years in cases of other diseases) and the severity of the vascular changes However it appeared as if the vascular changes in mitral disease were much more advanced in cases in which the disease had existed for more than 7 years Pronounced vascular changes were seen in 15 per cent of the cases of mitral stenosis of grade 2 and in 50 per cent of grade 3 Only 2 cases, neither of which had advanced vascular changes, could be assigned to grade 4

We also noted the occurrence of microscopic thrombi fresh or fibrously organized, in as many as 29 7 per cent of the entire material (in 23 cases with mitral disease, in 29 in the rest of the material) No distinct correlation was found between the occurrence of thrombi and the severity of the vascular changes, and the frequency of thrombi was not higher than in the controls Fresh thrombi were predominant in chronic lung congestion and in the control series (Gross lung emboli

were found in 17 per cent of patients with chronic lung congestion and in 14 per cent of the controls)

TABLE 3
*Cases Operated with Commissurotomy*

| Age and Sex | Duration of disease (before operation) | The day of operation | The day of death |
|---|---|---|---|
| 58 years man | 15 years | October-56 | 28 1 57 |
| 51 years man | 1 year | 16 2 59 | 26 2 59 |
| 49 years woman | 29 years | 4 3 59 | 5 3 59 |
| 52 years woman | 34 years | -54 | 16 2 60 |
| 57 years woman | 2 years | February-60 | 25 2 60 |
| 25 years woman | 6 years | 3 1 58<br>reop 3 6 61 | 3 6 61 |

The mitral disease series included 6 cases in which commissurotomy had been performed (Table 3) These cases invariably showed severe vascular changes of the type described above, including clear muscle hyperplasia of the media of the muscle arteries and the arterioles Four of the patients had died following operation, including one after a reoperation The primary commissurotomy in this case had been performed 3½ years previously, and the patient had since been symptom-free until she again had symptoms of cardiac failure in association with pregnancy She was reoperated upon in the 4th month of pregnancy One of the patients had died a few months after operation from complications (postop sepsis) In the remaining patient, who had lived for 6 years after the operation, the symptoms of cardiac failure recurred 2 months after the operation

DISCUSSION

The essential change in chronic lung congestion was found to be muscular hyperplasia of the media of the small arteries and arterioles, a finding in agreement with that of previous investigators (13) Intimal thickening was observed in most of the cases, usually in association with hyperplasia of the media, as was fibro-hyalinosis of the media These changes were of the same type as the vascular changes in the controls and seem, at least partly, to resemble the physiological changes of ageing (9, 19) But they were more advanced and occurred in lower age-classes in patients with chronic lung congestion

Some authors ascribe the changes in the small arteries and in arterioles entirely to vascular contraction (*O Neal, Thomas*) (23) without muscular hyperplasia, most authors ascribe it to muscular hyperplasia alone, while a third group describe both contraction and hyperplasia (*Short, Bayliss*) (27, 28) It is true that we found signs of vascular contraction but we feel that vascular contraction occurs in the initial stages of the disease, and that muscular hyperplasia soon develops and is hardly capable of regression This is supported, among other

things by the observations in our cases of commissurotomy (see Table 3) in which advanced muscular changes were seen both immediately and several years after the operation

In recent years it has been stressed that the vascular changes in mitral stenosis are much more pronounced in the lower lobes of the lung (*Doyle, Harrison*) (7, 10) We studied 10 cases in this respect (5 of mitral disease, 5 of chronic congestion of the lung of other origin) In all of these we found more advanced vascular changes (particularly muscular medial hyperplasia) in the lower lobes than in the upper ones, but sometimes the difference was only slight

Necrosis in the arterial walls, which has been reported in many publications (*Old, Russel* (22), *Hicks* (15)) was not seen in any of our cases, nor were signs of arteritis, described *interalia* by *Dammann* (6) Glomoid hyperplasia of the pulmonary vasculature described most frequently in pulmonary hypertension secondary to congenital heart disease (*Kuscko* (17), *Rossall* (25) *Waagenvort* (31), *Hruban* (16), *Brewer* (4), *Naeye* 21), *McCormack* (18), *Rutishauser* (26)) was not found in our material either Some authors have found a clear relation between thrombi and arteriosclerotic vascular changes in chronic lung congestion (*O Neal, Thomas* (30)) We could not confirm this Neither fresh nor old thrombi appeared to be more numerous in our cases presenting advanced vascular changes Moreover, we found thrombi to be equally common in our controls *Henry* (14) has, among other things, pointed out the relationship between the grade of mechanical stenosis of the mitral ostium and vascular changes in the lungs Our findings confirm this observations The severity of the vascular changes varied with the degree of stenosis

## SUMMARY


Vascular changes in lung congestion were studied in a necropsy material (100 cases of mitral disease, 75 cases of varying origin) The typical change was muscular hyperplasia of the media of the small arteries and arterioles The change was seen also in patients who had undergone commissurotomy for mitral stenosis, which argues against hyperplasia being due to vascular contraction alone In addition, the arteries and veins of varying calibre showed fibrous intimal thickening, and small arteries and arterioles [illegible]
changes were also [illegible]

[illegible] vascular changes


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29 de confrontation

30 *arteriosclerosis* lar hypertrophy

31 *Wage*

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# ANGIOTENSINASE CONTENT OF KIDNEYS OF NORMAL AND HYPERTENSIVE RATS

By

JENS BING

Received 27.xi.62

Quantitative determinations of the renal angiotensinase content have apparently been performed only by *Blaquier, Bohr, Taquini & Hoobler* (1961) who recently found no statistically significant difference between the values obtained in normal and renal hypertensive rats. The present investigation deals with similar studies on normal, renal hypertensive and desoxycorticosterone acetate plus salt treated rats. The renal renin concentrations of these animals have been published in previous papers (*Bing* 1962 a and b).

## MATERIAL AND METHODS

saline solution as drinking fluid.

*Methods.* Samples containing 0.5 ml of different concentrations (usually 0.05, 0.025

... angiotensinase was then stopped by boiling for 10 minutes and the residual angiotensin concentration was compared with that of a standard angiotensin solution. The *angiotensin assay* was performed by determination of the pressor response ...

... The renin assay was the same as described in previous papers (*Bing* 1962). Hypertension was induced by the method of *Byrom & Wilson* (1938).

Supported by grants from ... King Christian X's Foun-

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# ANGIOTENSINASE CONTENT OF KIDNEYS OF NORMAL AND HYPERTENSIVE RATS

*By*

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Received 27 vi 62

Quantitative determinations of the renal angiotensinase content have apparently been performed only by *Blaquier, Bohr, Taquini & Hoobler* (1961) who recently found no statistically significant difference between the values obtained in normal and renal hypertensive rats The present investigation deals with similar studies on normal renal hypertensive and desoxycorticosterone acetate plus salt treated rats The renal renin concentrations of these animals have been published in previous papers (*Bing* 1962 a and b)

## MATERIAL AND METHODS

The *material* included 56 kidneys from 41 albino rats of which 19 were controls 9 had unilateral partial constriction of the renal artery 7 were treated by subcutaneous injection of 5 mg of microcrystalline Percortene (Ciba) for about 4 months followed by injections of 2 5 mg per 100 g body weight twice a week and 4 were treated by both Percortene and clamping The last two groups received 0 9 per cent saline solution as drinking fluid

*Methods* Samples containing 0 5 ml of different concentrations (usually 0 05 0 025 and 0 0125 per cent) of kidney homogenates were incubated for half an hour at 37° C

s

... ... ... pared with that of a standard angiotensin solution The *angiotensin assay* was performed by determination of the pressor response

... ... ... assay was the same as described in previous papers (*Bing* 1962) Hypertension was induced by the method of *Byrom & Wilson* (1938)

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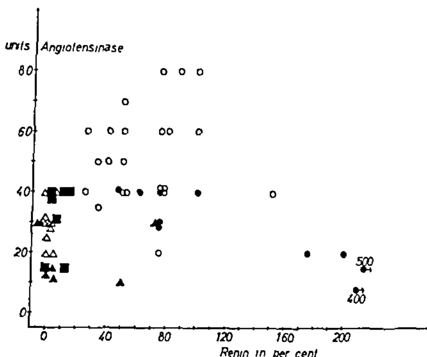



*Fig 1*

The figure shows that the renal angiotensinase content is lower in renal hypertensive and in desoxycorticosterone acetate plus salt treated rats than in normal controls. The decrease in angiotensinase content is seen to be non related to the highly variable renin content in the kidneys of the clamped and/or hormone treated rats

Normal rats ○
Unilaterally clamped rats ● clamped kidney ■ untouched kidney
DOCA plus salt treated rats △ nonoperated ▲ bilaterally clamped

## RESULTS AND DISCUSSION

The results are presented in Fig 1, which shows that while the renal angiotensinase content of normal rats was in the range 40 to 80 units *in all but two cases, both renal hypertensive and desoxycorticosterone* acetate plus salt treated rats had lower concentrations between 7 and 40 units. No significant differences in angiotensinase content were found amongst 1) the clamped and 2) the untouched kidneys of unilaterally clamped rats, 3) the kidneys from hormone treated rats and 4) the kidneys from clamped rats receiving hormone treatment. The low values obtained both in the renal hypertensive and in the hormone treated rats were found to be independent of the highly variable renin concentrations. The lack of a relation between the concentrations of angiotensinase and renin, which is seen in fig 1, was found also, when the concentrations in the two kidneys from single rats were compared. Out of 16 animals 10 had equal angiotensinase concentrations in both kidneys, in the remainder the concentration was higher in one kidney than the other by a factor 1.5 in 3 cases, by a factor 2 in 2 cases, and by a factor 5 in the case of a single unilaterally clamped animal in

which the untouched kidney contained 5 times more angiotensinase but 100 times less renin than the clamped kidney In other unilaterally clamped rats the angiotensinase concentration was the same in the two kidneys while the ratio between the renin concentrations was highly increased

The reasons for the differences between the results of the investigations of *Blaquier et al* and those reported here, with regard to both renal renin and angiotensinase content, are unknown They may partly be caused by different conditions during homogenation and incubation, as it is probable that more than one enzyme in the kidney has the property of destroying angiotensin (*Braun Menendez et al* 1946)

## SUMMARY

The renal angiotensinase content was found to be lower in renal hypertensive and in desoxycorticosterone acetate plus salt treated rats than in normal controls (Fig 1) The decrease in angiotensinase content was not related to the highly variable renin content in the kidneys of the clamped and/or hormone treated rats

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# AN ELECTRON MICROSCOPIC STUDY ON THE MORPHOLOGY OF THE ELASTIN IN FOETAL, HUMAN AORTAS

*By*

JØRGEN GARS JENSEN

Received 2 vii 62

In a previous study "Histochemical studies on elastic membranes of fetal human aortae" (4) argyrophile, PAS-positive fibres, which were localized in a matrix of acid mucopolysaccharides, were found as early as in the 2nd foetal month Elastic fragments were noted about the middle of the 3rd foetal month, and continuous, elastic "membranes" were demonstrable in about the 4th foetal month In addition the argyrophile fibres and the elastin were found to be closely interrelated and consequently it was discussed whether the argyrophile fibres might not form a direct part of the structure of the elastin, viz as a morphological component

The object of the present study has been to carry on these investigations into the morphology of elastin and argyrophile fibres using electron microscopy

Elastin is referred to but rarely in the electron microscopic literature References published in the previous study shall apply also here (4) Some of the authors cited advocate the presence in elastin of an internal, fibrillar structure *Hall et al* (3) treated elastin in human, aortas by elastase and found a network of irregularly anastomosing fibres with internal, fibrillar structures, the diameter of fibrils being about 200 Å

*Rhodin* examined elastin in lamina propria of the tracheal mucosa of rat (6) and reported the finding of longitudinally arranged fibrils in elastin, the diameters of fibrils being 70 100 Å

*H E Karrer* published an electron microscopic study on the structure of the connective tissue in the tunica propria of bronchioles (5) this author describes elastin as a dense mass of irregular size, shape, and surface, without periodicity, not readily observable except if prepared by opaque matter as e g phosphotungstic acid, the author observed occasional, fibrillar structures, the diameters of fibrils being about 110 Å

Other investigators have considered elastin as an amorphous substance *Cox & Little* (2) examined elastin hydrolysed from human tissue rich in elastin, the authors concluded that elastin in such tissue forms an irregular, three dimensional network composed of anastomosing, highly differently calibrated strands and fibres, and besides that sub microscopically the elastin is a homogenous, amorphous substance

The argyrophile fibres which are arranged in close relation to elastin in foetal human aortas were described in detail in the previous histochemical study (4) *Karrer* (5) and *Woodside* (8) in electron microscopic studies demonstrated a very close interrelation of elastin and collagen fibres *Karrer* provided his study (5) with several illustrations (Figs 7, 8, and 22) in which the fibres—by this author described as collagen fibres—are encircled by an elastic substance

## MATERIAL AND TECHNIQUE

Aortas from 10 human foetuses have been examined the lengths of foetuses ranging from 5 to 23 cm All foetuses were alive at the time when they were removed by h[illegible]

[illegible] this method has been found to remove all other structures but elastin (4)

The s[illegible]
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pendicu[illegible] axis of the gelatine capsule thus it is possible to place the section pyramid close to the lumen of the aorta thereby warranting the correct localization in the tunica media Some of the specimens are impregnated by 10 per cent phosphotungstic acid others by uranylacetat 2 per cent Thickness of cuts about 400 A

## RESULTS

Low electron microscopic magnification of the tunica media from aortas in human foetuses presents a morphological picture much reminding of the one known from optical microscopic studies (4), viz

A moderately cellular tissue with the characteristic, oblong deeply notched nuclei an irregularly dispersed protoplasm, occasionally the nuclei are thinlycoated *but also long* not infrequently slender, rami are seen generally there is no distinct line of demarcation against the intercellular structure Such low magnification presents two, morphologically deviating, intercellular structures (Fig 1) A greyish, apparently amorphous substance arranged in irregular, partly coherent, partly fragmented strands forms an intercellular network Usually however it is manifest as strands parallel to the lumen of the vessel As to shape and localization this substance corresponds to the histochemical elastin In close apposition to the elastin fasciae of fibres are noted often in direct continuation of the substance of elastin

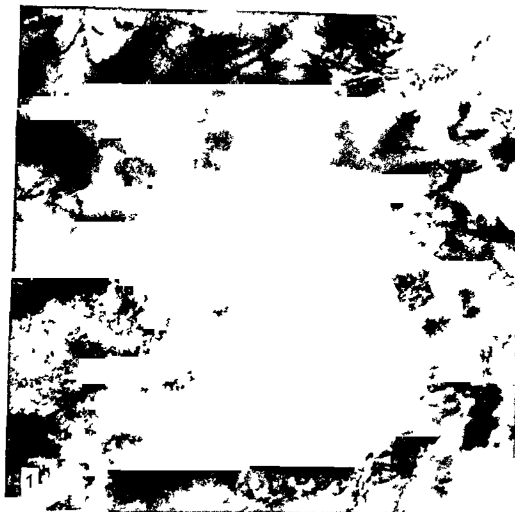


*Fig. 1*

Cross section of the aorta media from a foetus measuring 11 cm (× 24 000). Fibroblast encircled by elastin and argyrophile fibres. Note the intertwining of fibres which are seen in intimate contact with elastin.

In such low magnification the fibres seem to be massive and also the structure seems to be more dense than that of elastin. No periodicity is observed. The morphological localization of these fasciae of fibres is similar to the one known from the histochemical argyrophile fibres (Figs. 1–5).

Neither collagen fibres nor reticulin were observed in the tunica media of the examined aortas from foetuses of 23 cm or less.

*Using higher magnification and impregnation by phosphotungstic* acid and uranyl the structure and interrelation of elastin and argyrophile fibres is further examined.

It will be noted how the fibrillar substance in close apposition to the elastin and hence corresponding to the histochemically determined argyrophile fibres are composed of fasciae of fibres, the diameters of which range between 350 and 400 Å. By crosssection the fibres will be




*Figs* 2–5

*Fig* 2 Foetus measuring 14 cm (× 41 500) To the left elastin to the right argyrophilic fibres Note the very close interrelation of elastin and argyrophile fibres

*Fig* 3 4 5 Foetus measuring 14 cm (× 50 000 78 000 and 63 000) The intertwined argyrophile fibres (350–400 Å in diameters) are seen in fasciae The fibres are curved and intertwined they seem to be spiral shaped

circular, in other planes their courses are *noted to be more less curved*, generally they are arranged in small fasciae, not infrequently they have intertwined, rather giving an impression of having a shaped course

In phosphotungstic acid preparation the fibres are rich in opaque matter although the degree of opacity may vary, if the degree of opacity *be low the fibres will* present an internal, fibrillar structure Using very high magnification the internal structure of the 350 Å wide fibres is seen to be composed of fibrils the diameters of *which are 30–50 Å* (Figs 6, 7) These latter present a curved, tortuous course resembling intertwined spirals, their outlines may be more or less blurred, and in very high magnification also these fibrils present internal, fibrillar structures (Fig 9)

*Thus the argyrophile fibres with diameters* approximating *350 Å* are curved, their course seems to be spiral shaped, and they have intertwined with several other fibres thereby forming fasciae The internal structure is fibrillar and these fibrils as well, the diameters of which are 30 50 Å, are curved and intertwined, occasionally actual, intertwined spirals are seen distinctly In high magnification even the spiral shaped fibrils with diameters of 30–50 Å seem to have an internal, fibrillar *structure All of these systems of spirals may be more or less* densely intertwined

The elastin, which in low magnification was found to be an apparently amorphous substance, presents quite a different picture in higher magnification The degree of opacity of the grey substance varies, quite often the opacity may be most *marked peripherally in an elastin* "islet", but the internal, fibrillar structure is demonstrable all over the cut surface (Figs 2, 8) Curved, often distinctly spiral shaped fibrils with diameters of about 30–50 Å are seen to be intertwined thus forming a network (Fig 8) The density with which they are packed varies thereby determining the fluctuating degree of opacity of the elastin When densely packed they form fibre-mimicking structures which are un*distinguishable from* that of the argyrophile fibres (the order of magnitude being 350 Å) the internal fibrils of which are less densely packed

Not infrequently well-defined argyrophile fibres, the order of magnitude of which is 350 Å, may be demonstrable centrally in typical elastic substance, (Figs 2, 4) fibrils, the order of magnitude of which ranges *between 30 and 50 Å*, are seen *quite often* to pass from the elastin into direct continuation of the internal fibrils of the argyrophile fibrils (Figs 8, 9)

Suppose an incision be made through a fascia of "swollen" closely

*Figs 6 7*

*Fig 6* Foetus measuring 11 cm (× 152 000) Fasciae of argyrophile fibres (350 400 Å *in diameters*) with internal fibrillar structure of curved fibrils (30 50 Å in diameters) occassionally markedly spiral shaped

*Fig 7* Detail of Fig 6 (× 324 000)


6

circular, in other planes their courses are noted to be more less curved generally they are arranged in small fasciae, not infrequently they have intertwined, rather giving an impression of having a shaped course

In phosphotungstic acid preparation the fibres are rich in opaque matter although the degree of opacity may vary, if the degree of opacity be low the fibres will present an internal, fibrillar structure Using very high magnification the internal structure of the 350 Å wide fibres is seen to be composed of fibrils the diameters of which are 30 50 Å (Figs 6, 7) These latter present a curved, tortuous course resembling intertwined spirals, their outlines may be more or less blurred, and in very high magnification also these fibrils present internal, fibrillar structures (Fig 9)

Thus the argyrophile fibres with diameters approximating 350 Å are curved, their course seems to be spiral shaped, and they have inter twined with several other fibres thereby forming fasciae The internal structure is fibrillar and these fibrils as well, the diameters of which are 30–50 Å, are curved and intertwined, occasionally actual, inter twined spirals are seen distinctly In high magnification even the spiral shaped fibrils with diameters of 30–50 Å seem to have an internal, fi brillar structure All of these systems of spirals may be more or less densely intertwined

The elastin, which in low magnification was found to be an appa rently amorphous substance, presents quite a different picture in higher magnification The degree of opacity of the grey substance varies, quite often the opacity may be most marked peripherally in an elastin "islet", but the internal, fibrillar structure is demonstrable all over the cut sur face (Figs 2, 8) Curved, often distinctly spiral shaped fibrils with diameters of about 30–50 Å are seen to be intertwined thus forming a network (Fig 8) The density with which they are packed varies there by determining the fluctuating degree of opacity of the elastin When densely packed they form fibre-mimicking structures which are un distinguishable from that of the argyrophile fibres (the order of magni tude being 350 Å) the internal fibrils of which are less densely packed

Not infrequently well-defined argyrophile fibres, the order of magni tude of which is 350 Å may be demonstrable centrally in typical elastic substance (Figs 2, 4) fibrils the order of magnitude of which ranges between 30 and 50 Å, are seen quite often to pass from the elastin into direct continuation of the internal fibrils of the argyrophile fibrils (Figs 8, 9)

Suppose an incision be made through a fascia of "swollen" closely

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*Figs. 6–7*

*Fig 6* Foetus measuring 11 cm × 152 000 Fasciae of argyrophile fibres (350 400 Å in diameters with internal fibrillar structure of curved fibrils (30 50 Å in diameters occassionally markedly spiral shaped

*Fig 7* Detail of Fig. 6 × 224 000



packed, argyrophile fibres, the picture thus obtained were undistinguishable from the one of "elastin"

In sections from hydrolysed aortas, which from a histochemical point of view represent pure elastin only, low magnification will demonstrate a presence of strands of a greyish, apparently amorphous substance with varying opacity, the periphery may be the site of more dense, *fibre-mimicking* structures A presence of cells was never observed

In high magnification of hydrolysed medial aortas elastin discloses a network structure of fluctuating *opacity, its aspects being the same* as the one known from ordinary electron microscopic sections The network is composed of spiral shaped fibrils, the diameters of which range between 30–50 Å The density with which these spirals intertwine may vary, if densely intertwined they will be curved (spiral shaped) fibres with diameters of about 350 Å, they will be of the same aspect as argyrophile fibres (Figs 10, 11)

## DISCUSSION

Using electron microscopy structures have been identified in human, foetal tunica media of aortas the morphology of which tally with histochemical elastin and argyrophile fibres In electron microscopic pictures such structures as correspond to the histochemical, argyrophile fibres, have been found to be composed of fasciae of curved (*spiral shaped*), intertwined fibres with diameters of about 350–400 Å Even these fibres *present an internal arrangement of* spiral shaped, intertwined fibrils the diameters of which range between 30 and 50 Å The intertwining of the fibrils may be more or less dense, if densely packed these fibrils form a well defined, argyrophile fibre the diameter of which range between 350 and 400 Å If less densely packed the aspect of the argyrophile fibre *will be of a looser, rather "swollen" nature, the outlines* somewhat blurred If several fibres of this category be arranged closely *together, the fibrillar network thus formed will be identical to the one* found in elastin

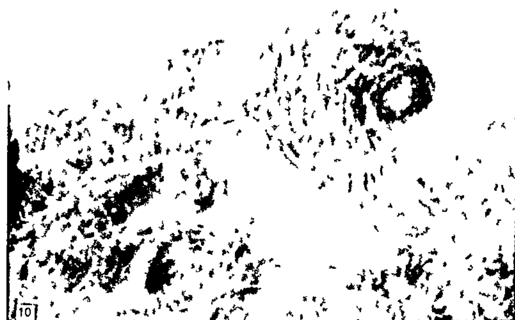
The following theory seems not improbable "elastin" and argyrophile fibres might represent two different, functional stages of the elastic tissue elastin Indeed the theory is supported by the fact that structures, *the morphology of which is identical to that of argyrophile* fibres and "elastin", are found in the hydrolysed aortas, *i e* in histo-

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*Figs 8 9*

*Fig 8* [illegible] 21 cm (× 257 000) At the top the elastin is present as a argyrophile [illegible]

*Fig 9* Detail of Fig 8 (× 545 000) Note the internal fibrillar structure of the fibrils with a diameter 30 50 Å





11

*I gs 10 11*

I ct is mca t r ng cm × 39 000) A rta is I lr ly I j ri r t f xati n an I m I t ling I la tin at var o stag N te the j ral I aje I fil res (350 400 A in lia m ters) with i t ral tru t i of sj ral sl aje I f I r ls (30 0 A in I neter

chemically and biochemically pure elastin More or less "swollen", argyrophile fibres passing into direct continuation of the fibrils of elastin are not ingrequent, they represent the functional, intermediate stages

At present it can hardly be decided whether the individual elements represent one or the other functional stage *although it might be relevant* to assume that the argyrophile fibres account for the resting phase

Neither collagen fibres nor any other fibres with periodicity—as observed by *Karrer & Woodside* in relation to elastin in bronchioles—were demonstrable in the here discussed tunica media in foetal, human, aortas Several of the fibres which *Karrer* has found to be in close apposition to elastin (in bronchioles) (5), (Figs 7, 8, and 22)) and to be characterized as being of a collagen nature, presented an internal structure with a curved course and hence, were reminding of argyrophile fibres

The fluctuating opacity of elastin, as described by several investigators and also observed in the present study, may be explainable on the basis of the here discussed working theory, always remembering that dense intertwining of the individual fasciae of spirals provides well defined, opaque, spaced fibres Conversely, a swelling of these fasciae will provide fibrils with faint opacity spaced by small quantities only of an amorphous substance which in sections present an amorphous, greyish substance The studies on elastin shall be continued with a view to further elucidating the morphology of such systems of spirals

## CONCLUSION

The presence of an internal (spiral shaped), fibrillar structure has been demonstrated in elastin from the tunica media of foetal, human aortas A fibrillar structure has been demonstrated in close apposition to elastin the internal, fibrillar structure of which is curved and tortuous (spiral shaped) and thus identical to the internal, fibrillar structure of elastin It has been suggested that fibrils which as to localization correspond to histochemical, argyrophile fibres, actually represent functional stages of elastin Hence it may be assumed that elastin has a systematic well arranged internal spiral shaped, fibrillar structure, the composition of which determines its elasticity

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3 *Hal*[illegible]
T[illegible]
4 [illegible]



*Figs 10 11*

Foetus measuring 5 cm (× 390 000 Aorta is hydr lysed pri r t fixati n an l im
bedding Elastin at vari us stage Note the spiral shape l fibres (350 400 Å in dia
meters) with internal structures of spiral shaped fibrils (30 50 Å in diameters)

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*FROM THE UNIVERSITY INSTITUTE FOR EXPERIMENTAL MEDICINE COPENHAGEN Ø DENMARK HEAD JENS BING M.D*

# ACUTE VASCULAR DISEASE CAUSED BY SEVERE RENAL ISCHAEMIA

*By*

JØRN GIESE

Received 3 vii 62

Many studies have demonstrated the close association between reduction of renal blood supply and development of necrotizing vascular disease (e g *Goldblatt* 1938, *Winternitz et al* 1940) Several links in the chain of events initiated by the induction of kidney ischaemia still remain unknown, and the question of the relative importance of different factors in the pathogenesis of vascular necrosis cannot be considered finally settled

The present paper reports studies in rats exposed to severe renal ischaemia, the interest has been centered on changes in vascular permeability and the occurrence of deposits of serum proteins in the vascular walls Attempts at modification of the pathological manifestations by administration of quinine are presented, this part of the study being inspired by reports on the effectiveness of quinine as an anti-permeability compound in various forms of experimentally increased capillary permeability (*Carone & Spector* 1960, *Spector & Willoughby* 1960)

## MATERIAL AND METHODS

*Animals* Albino rats mostly female weighing approximately 200 grammes were used

*Methods for induction of kidney ischaemia* In a smaller number of animals the method decribed by *Loriner & Goracz (1954)* for induction of experimental hyper tension was used in a modified form Rubber capsules were applied to both kid neys contrary to the *original* method care was taken to insure extreme renal ischaemia by very tight application of the capsules Although productive of lesions the method was found unsuitable because of difficulties in standardizing the pres sure applied to the kidneys

The method adopted next was found to be more reproducible Clamps made of silver wire internal *width* 0 15 *mm* were applied on both renal arteries This method produced pathological manifestations in a very high percentage of animals when applied to rats weighing about 200 grammes

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In studies concerning the possible effect of quinine it was felt necessary to eliminate variations in fluid intake, a *standard procedure* was adopted. Silver clamps were applied on the renal arteries, 5 ml of saline were injected subcutaneously at the time of operation and again 5 hours later. No drinking water was provided and the animals were killed 24 hours after operation.

*Quinine* was used as a 10 per cent solution of quinine hydrochloride dissolved in a 10 per cent solution of urethane. Doses of 125 mg per kg body weight were injected subcutaneously at the time of operation and again 5 hours later.

*Morphologic methods.* At autopsy effusions were collected by a pipette and volumes measured. Tissues were fixed in calcium chloride formalin as a routine. In some cases Carnoy's or Regaud's fixation fluid was used. Periodic acid—Schiff staining was employed as a routine (*Pearse* 1960). Selected tissue blocks known to contain pathological vessels were sectioned for special stains: *Casons* stain (1950), Mallory's phosphotungstic acid—haematoxylin, haematoxylin—eosin and *Feulgens nuclear stain, alternate PAS—stained sections being used for comparison.*

In evaluation of vascular pathology lesions were graded using a scale ranging from 0 to +++, the average degree of vascular disease for an animal was calculated on the basis of several slides and *expressed as absent, minimal,* moderate or severe.

*Experiments with fluorescent protein tracer.* Pooled rat serum was conjugated with Lissamine Rhodamine RB 200 as described by *Nairn* (1962). Unconjugated dye was removed by charcoal extraction. Various doses of tracer and different routes of administration (intravenous and intraperitoneal injection) were tried. In all experiments a sham operated control animal and an animal subjected to kidney ischaemia were injected with identical amounts of tracer protein. After fixation in formalin or Carnoy's fluid tissues were embedded, deparaffinized sections mounted in "Fluormount" (I. Gurr) were examined under ultraviolet-blue light. *Alternate PAS stained sections were used for comparison.*

Two different batches of conjugated serum were used. Serum A (2 experiments): Protein concentration about 40 mg/ml. Molecular ratio of fluorochrome to protein about 3. Total dose employed 3.4 ml.

Serum B (3 experiments): Protein concentration about 20 mg/ml. Molecular ratio of fluorochrome to protein about 0.5 (suboptimal degree of conjugation). Total dose employed 8.16 ml.

Both sera were compared with a normal unconjugated rat serum by immunoelectrophoresis. Serum B showed a seemingly normal precipitation pattern, whereas serum A which was subjected to immunoelectrophoresis several months after the experiments employing *this serum, showed a tendency to double contouring* of the albumin precipitation line: this may possibly indicate a slight denaturation.

*Protein concentrations* in sera and effusions were determined by spectrophotometric measurements at 2600 and 2800 Å. A nomograph (F. Adams) based on the data of *Warburg & Christian* (Biochem. *Z. 310,* 384, 1942) was used.

*Immunoelectrophoresis* was performed as described by *Scheidegger* (1955). Apparatus LKB 6800 A. Antiserum was produced in rabbits by repeated injections of rat plasma mixed with Freunds adjuvant.

*Paper electrophoresis* was performed in LKB apparatus 3276 BN (horizontal strips) with Schleicher and Schull 2043 bmgl paper strips. Buffer: veronalbuffer *pH 8.6, 0.07 molar with 5 mg %* calcium (*Laurell* 1956). The dried strips were stained with Amidoblack 10 B.

*Serum urea* was determined by Conway's method.

## RESULTS

### *Clinical Picture*

Animals subjected to kidney ischaemia by the methods described are generally in a bad condition after 24 hours: their general condition seems clearly worse than the condition of animals 24 hours after bilateral nephrectomy. Very often dyspnoea is a prominent feature.

In animals *subjected* to the standard procedure only small changes in body weight were recorded in most cases. Measurements of blood

TABLE 1

*Autopsy Observations, Chemical and Histological Studies in 10 Control Animals and 10 Quinine-Treated Animals*

| | Rat nr | Effusion volume (ml) | | Protein conc (g%) | | Serum urea | Pulmonary oedema | Oedema of pancreas | Occurrence of PAS positive vascular deposits | | | | Average degree of vascular disease |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Pleural cavity | Peritoneal cavity | Effusion fluid | Serum | | | | Pancreas and duodenum | Intestines | Stomach | Liver | |
| Control animals | 612 | 1.0 | 0 | 3.5 | 6.6 | 294 | +++ | ++ | (+) | (+) | (+) | 0 | minimal |
| | 616 | 5.0 | 3.0 | 2.7 | 7.8 | 250 | 0 | +++ | ++ | +(+) | 0 | 0 | severe |
| | 617 | 3.0 | 0 | 2.9 | 7.4 | 268 | 0 | +++ | ++ | + | 0 | 0 | severe |
| | 622 | 3.0 | 0.5 | 2.3 | 7.0 | 299 | +++ | +++ | ++ | ++ | 0 | + | severe |
| | 623 | 1.5 | 1.5 | 3.1 | 7.7 | 290 | 0 | +++ | ++ | ++ | 0 | necroses | severe |
| | 627 | 3.0 | 0.5 | 1.5 | 6.3 | 363 | 0 | ++ | + | (+) | 0 | 0 | moderate |
| | 629 | 0.5 | 0.1 | 2.3 | 4.8 | 321 | 0 | ++ | + | + | 0 | 0 | moderate |
| | 630 | 0 | 0 | — | 6.7 | 314 | 0 | + | + | ++ | 0 | (+) | severe |
| | 632 | 5.0 | 0 | 1.7 | 7.2 | 240 | +++ | ++ | + | 0 | 0 | 0 | moderate |
| | 634 | 3.5 | 0.3 | 4.4 | 6.4 | 276 | 0 | +(+) | (+) | (+) | 0 | (+) | minimal |
| Quinine-treated animals | 611 | 0.1 | 0 | — | 6.6 | 278 | 0 | (+) | 0 | 0 | 0 | 0 | absent |
| | 613 | 0 | 0 | — | 6.3 | 299 | 0 | 0 | 0 | 0 | 0 | 0 | absent |
| | 621 | 0 | 0 | — | 6.7 | 284 | 0 | + | (+) | ++ | 0 | 0 | severe |
| | 624 | 0.1 | 0.1 | 3.2 | 6.0 | 286 | 0 | + | ++ | ++ | (+) | (+) | severe |
| | 628 | 0 | 0 | — | 6.8 | 280 | 0 | 0 | 0 | 0 | 0 | 0 | absent |
| | 631 | 0.4 | 0 | 2.6 | 5.7 | 248 | 0 | (+) | + | +(+) | 0 | 0 | severe |
| | 633 | 0.2 | 0 | 1.5 | 5.8 | 241 | 0 | (+) | (+) | 0 | 0 | 0 | minimal |
| | 636 | 0 | 0 | — | 6.5 | 288 | 0 | (+) | (+) | 0 | 0 | 0 | minimal |
| | 637 | 0 | 0 | — | 6.9 | 253 | 0 | ++ | (+) | +(+) | 0 | 0 | moderate |
| | 638 | 0 | 0 | — | 6.3 | 246 | 0 | (+) | 0 | ++ | 0 | 0 | moderate |

pressure (plethysmographic method) were performed in a few animals, but no conclusive results were obtained Anaesthesia was badly tolerated, and it was difficult to obtain good readings in unanaesthetized animals Bleeding by decapitaton was very sluggish in most cases

### *Autopsy Observations and Chemical Studies*

In most animals effusions in pleural and/or peritoneal cavities were found The colour of the fluid was varying from grayish—yellow to haemorrhagic In ten animals subjected to the standard procedure an average of 3 7 ml was found, one animal had no effusion and one had 9 ml of fluid

Determinations of protein concentration were performed on serum and effusion fluid from these animals, individual values are given in Table 1 (see below) Generally, the serum protein level was higher in animals showing large effusions Serum and effusion fluid from four animals were studied by immunoelectrophoresis and paper electrophoresis All protein fractions of serum could be identified in the effusion fluid The presence of fibrinogen in the effusion fluid was evident by the formation of clots after removal from the serous cavities

In many cases a pronounced oedema of the pancreas was noticed, in severe cases the pancreatic tissue looked like embedded in a gelatinous mass with widely separated lobules In some cases haemorrhages in the pancreas were found The tissues, especially the mesenterium, gave an impression of succulence Oedema of the lung was macroscopically visible in 3 out of 10 animals subjected to the standard procedure

Clearly visible necroses of the liver were found in a single case The kidneys were markedly changed, an intermingling of congested and pale areas on the kidney surface was the general finding

### *Microscopical Observations*

The most important feature was a deposition of PAS-positive substance in the walls of small arteries and arterioles These deposits were sometimes located in the whole circumference of the vessel (Fig 1), sometimes only in part of the circumference (Fig 2) In vessels longitudinally sectioned this patchy character of the deposition was often evident Vessels showing deposits were most often found in the pancreas and intestines, including the mesenterium, but were found also in the stomach and liver In rare cases deposits were found in arterioles in the right side of the heart In sections stained by Cason's trichrome the deposits showed up as red (fuchsinophilic) With Mallory's phosphotungstic acid-haematoxylin many PAS-positive deposits showed a dark blue reaction (fibrin reaction), deposits were eosinophilic in haematoxylin-eosin stained sections In places where PAS-positive deposits were found, a loss of nuclei in the media of the vessel was most often observed



Fig 1 Fig 2

*Fig 1* (PAS stain) Oedema of pancreas, arteriole showing PAS positive deposit in media (× 150)
*Fig 2* (PAS stain) Pancreatic artery showing PAS positive material in the media, only a part of the circumference is involved (× 375)

Another important finding was a pronounced interstitial oedema, especially noticeable in the pancreas Lobules of pancreatic tissue were widely separated (Fig 1), and a fibrillar precipitate was visible in the interstitium

The occurrence of these lesions varied in different animals, in severely affected animals many vessels showing PAS positive deposits could be found in a single section, in others only a few pathological vessels were found in several sections Interstitial oedema was likewise varying

The necrotic foci found in the liver of a single animal were rather sharply demarcated from surrounding, apparently normal, tissue

In sections of the kidneys a picture of severe degeneration was seen In some areas congestion was noted, other areas appeared totally ischaemic The degree of degeneration varied in different places, in some places no nuclei were visible, in others nuclei could still be found

*Studies Employing Fluorescent Protein Tracing*

These studies were undertaken in order to investigate, whether the PAS-positive deposits in vascular walls might be, at least partly, composed of serum proteins

As mentioned previously each of these experiments involved the administration of equal volumes of rhodamine-conjugated rat serum protein solution to a sham operated control animal and a rat subjected to kidney ischaemia Some difficulty was experienced in selecting suitable doses Five experiments were performed (10 animals), in four

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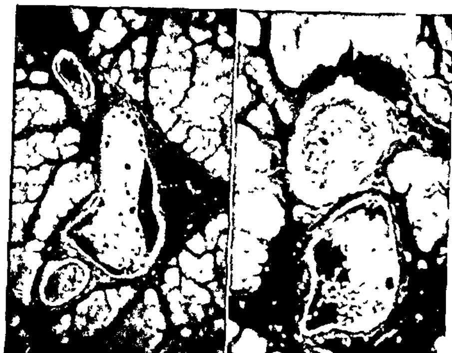


*Fig 3* *Fig 4*

*Fig 3* Fluorescence photomicrograph (ultraviolet-blue light) Section of pancreas showing large vein with tracer containing plasma in lumen At upper and lower left arterioles showing fluorescent tracer deposits in media (× 120)
*Fig 4* Fluorescence photomicrograph (ultraviolet-blue light) Pancreatic artery showing fluorescent tracer deposit in media (× 225)

experiments kidney ischaemia was induced by silver clamps and intraperitoneal injections of conjugated protein were used, in one experiment rubber capsules were applied to the kidneys and intravenous injections of tracer protein were used The experimental period covered 24–29 hours

In all of the five animals subjected to kidney ischaemia deposits of *tracer protein were found in the media of a smaller or larger number* of arterial or arteriolar vessels (Figs 3 and 4) These vessels were found in sections of pancreas or intestines, including the mesenterium In most places a good topographical correspondence between the fluorescent deposit seen under ultraviolet light and a PAS-positive deposit in the contiguous section examined under visible light was found In two of these experiments the tracer dose was apparently too small (the dye concentration of the conjugate was rather low), deposits were visible as rather *faint, but still well recognizable, yellow spots in these* cases, whereas the fluorescence was brighter and more orange red in the other experiments

*Care was taken to examine* a comparable number of sections from the control animal in each of the five experiments In no case were found any PAS-positive vascular deposits no fluorescent deposits were found in vascular walls in sections studied under ultraviolet light

All of the five animals subjected to kidney ischaemia showed a typical oedema of the pancreas in sections a fluorescent network of precipitated (fixed) protein in the interstitial tissue was seen

*Incompletely Operated Animals*

Two animals subjected to the standard procedure showed macroscopically visible intact areas in one kidney at autopsy These animals were in good clinical condition after 24 hours they showed a marked loss of weight (16 and 24 grammes) Serum protein was 8 2 8 3 per cent this value is higher than the values usually found in rats with complete kidney ischaemia and was judged to be due to dehydration No effusions were found one animal had no vascular lesions the other moderate lesions

*Nephrectomized Animals*

In order to study the effect of total lack of kidney function per se three animals were bilaterally nephrectomized but otherwise treated according to the standard procedure Clinical condition after 24 hours was good one animal had no effusion the two others had 0 2 and 0 5 ml of fluid in the pleural cavity Two of these animals had no vascular lesions and one animal minimal lesions

*The Effect of Quinine on Serous Effusions and Vascular Disease*

Quinine has been reported to suppress the increased capillary permeability following thermal or chemical injury (*Spector & Willoughby* 1960) Since the experimental disease described in the present paper appears to involve increased capillary permeability it was decided to try the effect of quinine on pathological manifestations resulting from kidney ischaemia

Twenty animals were subjected to the standard procedure Ten served as control animals ten animals were treated with quinine hydrochloride (125 mg/kg at the time of operation and again 5 hours later)

Clinically one striking difference between treated and untreated animals was observed dyspnoea was a general finding in the control animals whereas only two treated animals had slight dyspnoea

Results of chemical and morphological studies are given in Table 1 It is clearly seen that a very substantial reduction in volume of effusion fluid is obtained by treatment with quinine The average volume in the treated group is only a few per cent of the average value for the untreated control group Six out of 10 treated animals showed no effusions at all This absence or pronounced reduction of pleural effusions in the treated animals explains the clinical difference between the two groups Also oedema of the lung was found in three control animals but not in the treated group Generally oedema of the pancreas was much more pronounced in the control group



*Fig 3* *Fig 4*

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*Fig 4* Fluorescence photomicrograph (ultraviolet-blue light) Pancreatic artery showing fluorescent tracer deposit in media (× 225)

experiments kidney ischaemia was induced by silver clamps and intraperitoneal injections of conjugated protein were used, in one experiment rubber capsules were applied to the kidneys and intravenous injections of tracer protein were used The experimental period covered 24 29 hours

In all of the five animals subjected to kidney ischaemia deposits of *tracer protein were found in the media of a smaller or larger number of arterial or arteriolar vessels (Figs 3 and 4) These vessels were found* in sections of pancreas or intestines, including the mesenterium In most places a good topographical correspondence between the fluorescent deposit seen under ultraviolet light and a PAS-positive deposit in the contiguous section examined under visible light was found In *two of these experiments the tracer dose was apparently too small* (the dye concentration of the conjugate was rather low), deposits were visible as rather faint, but still well recognizable, yellow spots in these cases, whereas the fluorescence was brighter and more orange red in the other experiments

*Care was taken to examine a comparable number of sections from the* control animal in each of the five experiments In no case were found any PAS-positive vascular deposits no fluorescent deposits were found in vascular walls in sections studied under ultraviolet light

skin chemically induced pleurisy (*Spector & Willoughby* 1960), and some forms of proteinuria (*Carone & Spector* 1960) In the present study the administration of quinine brought about a complete or very substantial suppression of the formation of serous effusions This was thought to be due to an anti permeability effect of quinine, although it is realized that other effects (e g cardiovascular) of this compound might be of importance The effect of quinine on the occurrence of PAS positive deposits in arterial and arteriolar walls could not be considered to be significant

The present study offers a parallel to several observations in the literature Ligation of both main renal arteries in dogs causes necrotizing vascular disease and oedema of several organs (*Winternitz et al* 1940) In rabbits serous effusions are produced by bilateral infarctions of the kidneys (*Nairn* 1954) in further studies effusions were produced by administration of renal extracts and renin preparations to nephrectomized animals (*Nairn Masson & Corcoran* 1956) No vascular lesions were reported in these studies Partial renal infarctions in rats give rise to hypertension and vascular disease (*Loomis* 1946) In a few (unpublished) experiments using one of Loomis methods (infarction of one and a half kidney) I have noted a few animals displaying acute PAS positive vascular lesions in the pancreas these lesions have been found about two weeks after the operation and are very similar to the lesions produced by the methods described in the present paper although the adventitial cellular infiltration is more pronounced In two of these animals pancreatic oedema was present one had serous effusions The occurrence of pancreatic oedema in rats with experimental malignant hypertension has been described by *Byrom* (1958 59)

Deposits of fluorescent serum protein tracer in vascular walls have been demonstrated in rats exposed to repeated injections of angiotensin (*Giese* 1961) Exudation of serum proteins into vascular walls and other tissues has been stressed as an important pathogenetic factor in malignant hypertension (*Kincaid Smith McMichael & Murphy* 1958) and in human eclampsia (*Telford Govan* 1961)

## SUMMARY

1 Induction of severe renal ischaemia in rats gives rise to serous effusions interstitial oedema of connective tissue and acute vascular lesions involving deposition of PAS positive substances in arterial and arteriolar walls These pathological changes are manifest after 24 hours

2 Chemical studies show similarity between protein fractions of serum and effusions

3 Fluorescent protein tracer studies support the assumption that the deposits in arterioles and small arteries are formed (at least partly) by serum proteins

4 The central pathogenetic mechanism is supposed to be an increa

A rather large number of slides was examined in order to compare the vascular pathology in the two groups. It is seen from the table, that deposits were absent in 3 out of 10 treated animals, whereas all animals in the control group had PAS-positive deposits to a varying degree Seven treated animals showed deposits, the average degree of vascular disease in these animals may be a little less than the average for the untreated group Taking into account the small number of animals and the inherent difficulties of morphologic grading, the observed effect of quinine on the occurrence of PAS-positive deposits in vascular walls cannot be considered to be significant

## DISCUSSION

The experiments reported here show that induction of severe renal ischaemia in the rat may give rise to *increased capillary permeability* as evidenced by the formation of protein-containing effusions in the body cavities and interstitial oedema of connective tissue The protein fractions demonstrable in serum by immunoelectrophoresis and paper electrophoresis are also demonstrable in the effusion fluid, it seems that a greater transcapillary transport of plasmatic fluid is initiated by the induction of renal ischaemia

Another prominent pathological manifestation caused by renal ischaemia is a *vascular lesion*, which is characterized by deposition of a PAS-positive substance in the walls of small arteries and arterioles The studies employing fluorescent protein tracer support the assumption, that these deposits are composed (at least partly) of serum proteins the deposits are accompanied by a loss of nuclei in the media of the vessel

If these two pathological changes are related, a possible conclusion is, that the endothelial cell in the arteriole and small artery is affected in the same way as the capillary endothelial cell The increased permeability of the capillaries gives rise to serous effusions and interstitial oedema of connective tissue, whereas the increased permeability of the luminal endothelial layer in the arteriole or small artery may allow an increased passage of plasmatic fluid into the vascular wall, resulting in places in the deposition of proteins

The pathogenetic mechanism responsible for the increase in vascular permeability in animals subjected to kidney ischaemia has not been identified A release of renin from the ischaemic kidneys might be responsible, it is generally assumed, that such a release does occur and it is well known, that renin has the property of inducing increased capillary permeability (*Addis et al* 1949, *Paldino & Hyman* 1954)

Another possibly important mechanism might be an activation of plasma permeability globulins (*Spector* 1957)

Quinine has been shown to counteract increased capillary permeability in a number of experimental situations thermal injury of the

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# STUDIES ON C-REACTIVE PROTEIN

## 2 *The Presence of C-Reactive Protein during the Pre- and Neonatal Period*

*By*

LARS A HANSON and LARS A NILSSON

Received 17 ix 62

C-reactive protein (CRP) has been demonstrated in frequencies between 0 and 82 per cent during the latter part of the pregnancy (2, 5, 7, 8, 13, 15 16, 17, 18) and at parturition (2, 3, 4, 8, 10, 13, 15, 16, 18), but it is rarely found in umbilical cord sera (2, 8, 10, 15, 16, 18) except in a few investigations which report the frequency to be about 15 per cent (3, 4, 7, 19) These findings have been assumed to indicate that CRP does not pass the placental barrier (3, 8, 10, 15, 16, 19) CRP has not been demonstrated in human milk (10, 11) It has been discussed whether or not the infant may be able to form CRP shortly after parturition as in some cases CRP has been found in sera from infants during the neonatal period (3, 4, 7, 8, 10, 15, 16)

Recent studies have shown that the precipitation in agar gel is a very sensitive and specific method with which to demonstrate the presence of CRP (9) This method has been applied in a study on the presence of CRP in the serum and milk from the mother and in the serum from the offspring

### MATERIAL AND METHOD

*Human sera* Umbilical cord blood was taken from [illegible]

samples were obtained f [illegible]

[illegible] Stockholm

[illegible] with the blood samples from 15 [illegible] arturition

[illegible] antisera commercially available from Schieffelin Ltd were employed

*Method* The analyses were performed with the double diffusion in gel technique of *Ouchterlony* (12) in the micromodification of *Wadsworth* (20) A five basin arrangement was used as shown in an earlier paper (9)

sed permeability of endothelial cells *in* capillaries *and* in arterioles and small arteries

5 Treatment with quinine was found to suppress the formation of effusions very definitely The effect of quinine on the occurrence of PAS-positive vascular deposits was doubtful

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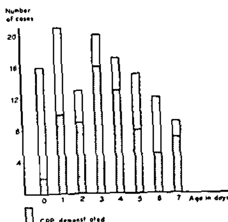



*Fig 1*

The presence of CRP in sera from hyperbilirubinaemic infants 0-7 days old as demonstrated with a diffusion in gel method



Fig 2

Comparative double diffusion analyses of a positive CRP control serum (upper middle basin) and two sera from infants one (upper left basin) and two (upper

precipitates crossed each other and identified with two precipitates in the spectrum formed by the control serum and CRPA (see Fig 2)

No inhibitory effect on CRP by umbilical cord serum could be noted in a control experiment in which a CRP-positive serum was incubated with a CRP negative umbilical cord serum prior to the diffusion-in gel analysis

## RESULTS

Analyses for the presence of CRP were performed in 46 pairs of maternal and umbilical cord sera taken after pregnancies of less than 40 weeks' duration CRP was found in only one of the umbilical cord sera, but was demonstrated in 38 of the maternal serum samples (Table 1) The umbilical cord serum which contained CRP was from a delivery taking place after 38 weeks of pregnancy In this instance CRP was also found in the serum from the mother There was no evidence of complication or disease in mother or child

TABLE 1
*Presence of CRP in Colostrum and in Sera from Foetuses and Neonates and the Corresponding Mothers*

| | | Number of samples | CRP | |
|---|---|---|---|---|
| | | | Positive | Negative |
| Pregnancy 15–38 weeks | Umbilical cord serum | 46 | 1 | 45 |
| | Maternal serum | 46 | 38 | 8 |
| Full term pregnancy | Umbilical cord serum | 58 | 3 | 55 |
| | Maternal serum | 58 | 51 | 7 |
| | Maternal colostrum | 15 | 0 | 15 |
| | Maternal serum | 15 | 15 | 0 |

Of the 58 umbilical cord sera from full term deliveries 3 contained CRP In the corresponding maternal sera CRP was present in 51 samples, but was not found in 7 (Table 1) None of the three positive cases showed any evidence of abnormality during pregnancy or parturition

CRP was not demonstrable in any of the 15 tested colostrum samples, whereas all of the serum samples from these mothers contained the C-reactive protein (Table 1)

Analyses of 16 serum samples taken on the first day of life from infants with jaundice showed, in agreement with the figures presented in Table 1, that CRP was present in the umbilical cord sera only in a very few cases (Fig 1) In serum samples taken one day after birth it was found in about 50 per cent of the cases A still higher frequency was found for the following three days, whereas it diminished at five and six days On the seventh day 7 of the 9 test samples showed the presence of CRP (Fig 1)

On double diffusion-in-gel analysis some of the sera from infants showed reactions of partial interference with the CRP of the control serum Many of the sera showed the presence of at least two separate precipitates when tested with CRPA In several instances two of these

In order to evaluate these findings a comparison should be made with the frequencies of CRP in sera from healthy neonates A recent investigation has shown that the frequencies at which CRP is present in healthy infants are slightly inferior to the ones found in this material consisting of hyperbilirubinaemic infants (1) This indicates that the demonstration of CRP in the first week of life cannot be used as a diagnostic criterion suggesting a presence of pathological conditions So far it remains unexplained at which stage the correlation between CRP and disease may be established in infants


## SUMMARY

The sensitive and specific double diffusion in gel method is employed for the investigation of CRP in the prenatal and neonatal periods

CRP is only occasionally found in umbilical cord sera whereas it is often found in maternal sera

CRP is present at increasing frequencies in sera taken during the first days of life from infants with hyperbilirubinaemia

In some of the cases these sera contained material giving a reaction of partial interference with the CRP in the control serum Some of the sera gave spectra demonstrating a heterogeneity of the CRP

The frequency at which CRP is found in the present material including infants with jaundice is almost similar to the one reported by the authors for healthy infants Thus the CRP test does not seem to be of any clinical value during this period of life



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## DISCUSSION

Studies on CRP in umbilical cord sera from prematures have been reported, among others, by *Valsecchi & Finzi* (19), *Bellomo & Muscolino* (3) and by *Bellomo, Muscolino & D'Urso* (4). The latter authors report the presence of CRP in all of their cases as demonstrated with the precipitation in capillary tube method This frequency for cord sera is not in accord with the one we have found with a diffusion-in-gel method It may be noted that a few of the precipitation reactions obtained with the capillary tube method can be demonstrated to be of a non-specific nature by means of diffusion-in-gel methods (9)

From reports in the literature (3, 4, 8, 15, 16, 19) as well as from our findings (1, 10) CRP seems to be present at a high frequency in the maternal sera at parturition but at a lower frequency in sera from the newborn Assumedly this may indicate that CRP does not pass the placental barrier Another possible explanation of these findings might be the presence of some kind of CRP-inhibiting substance in the umbilical cord serum On the other hand such a CRP-inhibiting effect of the umbilical cord serum could not be verified by control experiments

The analyses of sera taken from infants during their first week of life show the presence of CRP in more than 50 per cent of the cases *Nemir et al* (7), *Bellomo et al* (3, 4) and *Nesbitt et al* (8) among others, have reported the demonstration of CRP in some infants during the neonatal period *Bellomo et al* as well as *Nesbitt et al* obtained decreasing numbers of CRP-positive sera after the age of four to five days, which is in accord with our findings In a clinically thoroughly examined material, we have recently found a comparable distribution (1) The significance of the higher frequency of CRP-positive sera on the seventh day of the neonatal period is obscure but no definite conclusions may be drawn on the basis of such a small material It would be of interest to enlarge the material and extend the investigation to include more than the first week of life

The finding that CRP is seldom demonstrated in umbilical cord sera but is often found in sera from infants one or a few days old might indicate that infants are already capable of forming CRP when they are one day old It is possible that the fact of this condition may be a stimulus to the establishment of investigations on the mechanism and locale of CRP formation which at present are unknown (14) In this connection it is of interest to note that some of the sera from infants provided material which only gave a reaction of partial interference with the positive control serum Probably this illustrates the heterogeneity of the CRP (9), which was also demonstrated by the crossing of precipitates in CRP CRPA spectra (cf Fig 2 )

In several serum samples from neonates no CRP was found although all of these infants had hyperbilirubinaemia, in more than 2/3 of the cases to such an extent that exchange transfusions had to be undertaken

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# A FURTHER COMPARISON BETWEEN THE REITER PROTEIN COMPLEMENT FIXATION TEST (RPCF) AND THE TREPONEMA PALLIDUM IMMOBILIZATION TEST (TPI)

*By*

J KJELLANDER, O SIFVERS and Th M VOGELSANG

Received 26 v 62

In a previous paper (1) an investigation was described in which the RPCF was compared with the TPI in a selected material of sera from patients with and without evidence of luetic infection This investigation has been continued along the same lines

## MATERIAL

As before the sera were col [illegible]
tories for [illegible]
to find ser [illegible]
Sera given [illegible]

In all 7[illegible] sera were included in the present investigation 413 from Gothenburg and 31[illegible] from Bergen Thus together with the previous material 1257 sera have been tested

## METHODS

The same methods have been used in the present investigation as described earlier (1) The reader is referred to this paper for details Here it should be mentioned only that the sera were sent to the two laboratories without accompanying notes these were not produced until the examination had been completed

## RESULTS

The results are presented in Tables 1 8

Table 1 shows the outcome of the reactions in the whole material If the TPI is considered as specific, it will be seen from the table that the RPCF was positive in 583 out of 734 TPI-positive sera (79 4 per cent)

Table 2 shows the results from the part of the material which has been collected in the present investigation Of the [illegible] TPI
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16 of these sera came from patients with syphilis 13 of these were early, treated cases, where the TPI did never become positive or where the reaction had rapidly become negative 3 cases were old, treated cases In the remaining 27 cases evidence of syphilis was absent, and the RPCF must be considered as false positive in the majority of these cases, comprising 7 6 per cent of the RPCF-positive sera

Table 3 shows a comparison between the TPI and the STS in the present material *The table shows that only 254 out of the 402 TPI-positive sera (63 2 per cent)* gave clear-cut reactions with the STS, *i e* 15 per cent less than the RPCF In 46 of the 58 cases giving positive STS but negative or inconclusive TPI tests evidence of syphilis was lacking, and the STS has thus given false positive reactions in the majority of these cases, comprising 18 1 per cent of the STS positive sera The frequency of false positive reactors with the STS was thus more than twice that of the RPCF in this material

It should be pointed out in this connection that part of the material was selected on the grounds that the STS gave inconclusive results This explains the high frequency of inconclusive STS seen in Tables 3 5 and 7 Of course such a high number of inconclusive STS (40 7 per cent) will not be found in a non selected routine material

TABLE 4

*Outcome of RPCF and TPI in 165 Sera from Patients with Syphilis not Treated*
*i = inconclusive*

| TPI | RPCF | | | |
|---|---|---|---|---|
| | + | | i | Total |
| + | 135 | 12 | 7 | 154<br>93.3 % |
| — | 1 | 5 | 1 | 7<br>4 2 % |
| i | 2 | 1 | 1 | 4<br>2 4 % |
| Total | 138<br>83 6 % | 18<br>10 9 % | 9<br>5 5 % | 165<br>100 % |

The present material can be divided into two groups according to the presence or absence of evidence of syphilis 335 sera came from cases *presenting evidence of syphilis* Of these 165 were untreated and 170 were treated

Table 4 shows the outcome of the RPCF and the TPI in the untreated cases 93 3 per cent of the sera were positive with *the TPI* Of the 7 negative sera 2 came from patients with early syphilis where the RPCF was also negative 4 were classified by the clinician as latent syphilis on account of positive STS It is doubtful whether this diagnosis is correct since 3 of the sera gave negative RPCF and one gave inconclu-

TABLE 1

*Outcome of RPCI and TPI in 1257 Sera from Patients with and without Syphilis*
*i = inconclusive*

| TPI | RPCI | | | |
|---|---|---|---|---|
| | + | − | i | Total |
| + | 583 | 113 | 38 | 734<br>58.4% |
| − | 74 | 385 | 36 | 495<br>39.4% |
| i | 14 | 11 | 3 | 28<br>2.2% |
| Total | 671<br>53.4% | 509<br>40.5% | 77<br>6.1% | 1257<br>100% |

TABLE 2

*Outcome of RPCF and TPI in 728 Sera from Patients with and without Syphilis*
*i = inconclusive*

| TPI | RPCF | | | |
|---|---|---|---|---|
| | + | − | i | Total |
| + | 314 | 73 | 15 | 402<br>55.2% |
| − | 33 | 254 | 19 | 306<br>42.0% |
| i | 10 | 7 | 3 | 20<br>2.8% |
| Total | 357<br>49.0% | 334<br>45.9% | 37<br>5.1% | 728<br>100% |

TABLE 3

*Outcome of STS and TPI in 728 Sera from Patients with and without Syphilis*
*i = inconclusive*

| TPI | STS | | | |
|---|---|---|---|---|
| | + | | i | Total |
| + | 254 | 29 | 119 | 402<br>55.2% |
| − | 50 | 90 | 166 | 306<br>42.0% |
| i | 8 | 1 | 11 | 20<br>2.8% |
| Total | 312<br>42.9% | 120<br>16.5% | 296<br>40.7% | 728<br>100% |

TABLE 7

*Outcome of RPCF and STS in 170 Sera from Patients with Treated Syphilis*
*i = inconclusive*

| STS | RPCF | | | |
|---|---|---|---|---|
| | + | — | i | Total |
| + | 50 | 19 | 3 | 72<br>42 2 % |
| — | 14 | 28 | 2 | 44<br>25 9 % |
| i | 36 | 17 | 1 | 54<br>31 8 % |
| Total | 100<br>58 8 % | 64<br>37 7 % | 6<br>3 5 % | 170<br>100 % |

TABLE 8

*Outcome of RPCF and TPI in 474 Sera from Patients with Syphilis in Various Stages*

| Stages of syphilis | No of sera | Both + | TPI only + | RPCF only + | Both — |
|---|---|---|---|---|---|
| Prenatal | 17 | 15 | 2 | 0 | 0 |
| Early | 23 | 17 | 1 | 3 | 2 |
| Tertiary | 47 | 43 | 3 | 1 | 0 |
| Neuro | 51 | 50 | 1 | 0 | 0 |
| Latent | 126 | 105 | 10 | 3 | 8 |
| Treated | 210 | 120 | 38 | 16 | 36 |
| Total | 474 | 350 | 55 | 23 | 46 |
| Per cent | 100 | 73 8 | 11 7 | 4 9 | 9 7 |

The outcome of the TPI and the RPCF in the treated cases can be seen in Table 6 69 4 per cent of the sera were positive with the TPI and 58 8 per cent with the RPCF Presumably this difference is a reflexion of the known fact that the RPCF as a rule converts to negative more rapidly than the TPI after adequate treatment Since the material does not allow for a grouping as to year of treatment we can not say to which extent this is true in the present material

In table 7 the RPCF has been compared with the STS in this material of treated cases The table shows that the RPCF gives clearly positive reactions to a greater extent than does the STS the difference being about 16 per cent This, also is presumably due to the fact that the STS turns negative earlier than the RPCF after adequate treatment

In Table 8 a number of cases has been recorded in which data were available as regards the stage of the disease at the time when the patients were tested The table includes only those sera which gave clear cut results with both tests In all the TPI has given positive results more often than the RPCF, the difference being 6 8 per cent The

sive result with this reaction The last of the TPI-negative cases was classified as *aortitis luetica, giving positive STS and RPCF*

83 6 per cent of the sera were positive with the RPCF Of the 18 RPCF-negative cases, 3 came from patients with early syphilis two of which were also TPI negative 2 were classified as *neurolues* (TPI-positive) and 11 as latent lues (8 TPI-positive)

TABLE 5

*Outcome of RPCF and STS in 165 Sera from Patients with Syphilis not Treated*
*i = inconclusive*

| STS | RPCF | | | |
|---|---|---|---|---|
| | + | — | i | Total |
| + | 109 | 9 | 4 | 122<br>73 9 % |
| — | 4 | 4 | 2 | 10<br>6 1 % |
| i | 25 | 5 | 3 | 33<br>20 0 % |
| Total | 138<br>83 6 % | 18<br>10 9 % | 9<br>5 5 % | 165<br>100 % |

In Table 5 the RPCF and the STS are compared in this material of untreated syphilis It will be seen from *the table that the RPCF has* given about 10 per cent more clearly positive results than the STS, which to some extent has given inconclusive results The 9 cases giving *positive STS but negative RPCF comprise one case* of tertiary syphilis (TPI-positive), one case of neurolues (TPI positive) and 7 cases of lues latens (6 TPI-positive) Of the 4 cases which gave positive RPCF but negative STS, 2 were classified as prenatal lues (umbilical vein blood, TPI positive) and 2 as latent syphilis (TPI positive)

TABLE 6

*Outcome of RPCF and TPI in 170 Sera from Patients with Treated Syphilis*
*i = inconclusive*

| TPI | RPCF | | | |
|---|---|---|---|---|
| | + | | i | Total |
| + | 87 | 29 | 2 | 118<br>69 4 % |
| — | 11 | 33 | 2 | 46<br>27 1 % |
| i | 2 | 2 | 2 | 6<br>3 5 % |
| Total | 100<br>58 8 % | 64<br>37 7 % | 6<br>3 5 % | 170<br>100 % |

*FROM THE DEPARTMENT OF VIROLOGY NATIONAL INSTITUTE OF PUBLIC HEALTH OSLO NORWAY*

# GEL FILTRATION OF SE POLYOMA VIRUS HAEMAGGLUTININ ON SEPHADEX

*By*

J JONSEN, K HELGELAND and O LAHELLE

Gel filtration with the crosslinked polysaccharide dextran "Sephadex" has recently been widely used for the separation of substances with different molecular dimensions (1 3) The separation is related to the molecular size of the test substances and is only to a small degree influenced by the solvent However, with some substances the ionic strength and the pH of the gel column may cause both positive and negative sorption to the bed material partly caused by small amounts of ionized groups (2)

Gel filtration with sephadex has recently been described as a desalting method for virus preparations which contained salt as a result of purification procedures (4)

The present publication describes gel filtration experiments with polyoma virus suspended in a nutrient medium The experimental conditions have been varied by changes in the molarity and/or pH of the buffer used for equilibration of the sephadex columns

## MATERIAL AND METHODS

### *Virus Material*

The poly ma agent origina

wart It induced tumour form

embryo monolayer cultures c

and 0 002 per cent phenol rec

serum proteins the gel exper

[illegible] days before use the virus suspension was clarified by low speed centrifugation

### *Preparati n of Column*

Sephadex G 75 (Pharmacia Uppsala Sweden) with a water regain of 7 7 g water per g dry gel was employed 11 g of the gel was suspended in about 150 ml phosphate buffer of desired molarity and ionic strength After sedimentation for [illegible]

30 m n tes [illegible]

majority of these cases belong to the group of latent syphilis and, as expected, among the treated cases In the remaining group of late syphilis the difference is not as marked, and in the group of early syphilis there were 20 RPCF-positive sera but 18 TPI-positive

## DISCUSSION

The results of the present investigation do well agree with those of the previous one (1) and also with results obtained by other investigators, *e g* *Wilkinson* (2), *Olansky et al* (3), *Dorp-Petersen* (4) and *Vogelsang et al* (5) It is evident that the RPCF, as carried out here, cannot replace the TPI It does not attain the specificity of this reaction, but it can be considered as a valuable complement to the STS It is possible that a standardization and improvment of the Reiter antigen along the lines attempted by, *e g*, *Pillot et al* (6), will further increase the sensitivity and specificity of the reaction Probably it will not be long before it is justified to replace the cardiolipin antigen of the WR with a Reiter antigen

## SUMMARY

This investigation has shown that, as far as specificity concerns, the RPCF can be classified between the TPI and the STS In an undifferentiated material of syphilitic sera the RPCF gave 10–15 per cent more positive reactions than the STS, but about 10 per cent less positive reactions than the TPI The frequency of false positive reactions was about twice as high with the STS as with the RPCF (18 1 per cent as compared to 7 6 per cent) As a rule, RPCF turns positive somewhat earlier than the TPI in cases of early syphilis On the other hand, the TPI remains reactive for a longer time after treatment than does the RPCF which in this respect seems to occupy a place between the TPI and the STS

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precipitation with $AgNO_3$ No precipitation took place in the high molecular fractions (1 14) which contained the haemagglutinins The elution of chloride started in fractions in which the first appearance of small molecular UV material was evident The total UV material in these fractions was dialysable by conventional dialysis against 0 85 per cent saline

The experiment with the 0 001 M pH 7 2 buffer gave results nearly identical to those obtained with the 0 01 M pH 7 2 buffer The haemagglutinins were recovered in 33 ml (fractions 7–12) and the haemagglutinin peak was separated from the subsequent ultraviolet absorbing peak by six fractions as was the case with the 0 01 M pH 7 2 buffer

The experiment with the 0 001 M pH 5 5 buffer followed essentially the same pattern as that obtained with the 0 01 M pH 7 2 buffer The haemagglutinin containing fractions eluted with the acid buffer were slightly turbid A summary of results obtained in the filtration experiments are presented in Table 1

TABLE 1

*Gel Filtration of Polyoma Virus Suspensions with Sephadex G 75 as Bed Material*

| Buffer | Input | | Tube no | Recovery | | | |
|---|---|---|---|---|---|---|---|
| | HA total | OD total | | HA total | % | OD total | % |
| 0 01 M pH 7 2 | 6400 | 31 3 | 7 13 | 3307 | 52 | 9 7 | 31 |
| | | | 14 21 | 0 | 0 | 1 5 | 5 |
| | | | 22 40 | 0 | 0 | 22 7 | 73 |
| 0 001 M pH 7 2 | 3200 | 31 3 | 7 12 | 2035 | 0 | 9 6 | 31 |
| | | | 13 19 | 0 | 0 | 0 9 | 3 |
| | | | 20–34 | 0 | 0 | 20 9 | 67 |
| 0 01 M pH 6 5 | 2816 | 38 7 | 2 8 | 3216 | 114 | 30 6 | 79 |
| 0 01 M pH 6 0 | 1600 | | 8 13 | 1440 | 90 | | |
| 0 01 M pH 5 5 | 3200 | 35 4 | 7 12 | 1760 | 55 | 16 8 | 48 |
| | | | 13 19 | 0 | 0 | 1 6 | 4 |
| | | | 20–34 | 0 | 0 | 21 4 | 60 |

## DISCUSSION

Desalting of virus suspensions is mostly achieved by dialysis or by ultracentrifugation These methods are at best time consuming and may cause loss of virus material The gel filtration experiments described above show that sephadex G 75 is excellent for the desalting of SF polyoma virus which was present in a nutrient medium The small molecular substances which absorb ultraviolet light were eluted in fractions which were distinctly separated from the high molecular

column was kept in a distinct vertical position and was partly filled with phosphate buffer of room temperature when the sephadex suspension was introduced The outlet cock of the column was kept closed until a 5 cm layer had formed With the outlet cock open the suspending buffer was then allowed to drain The length of the prepared wet column was about 25 cm The column was then washed with 30 ml of phosphate buffer The volume of the prepared column approximated 125 ml corresponding to a capacity of about 35 ml for desalting purposes

*Application of Material*

The phosphate buffer overlaying the sephadex column was allowed to drain until it had reached the upper niveau of the sephadex particles 25 ml of virus suspension (PP 39) was then carefully layered upon the column

A small amount of buffer was used to wash the inner surface of the glass column after the virus suspension had entered the sephadex column It was followed by a larger volume of phosphate buffer for elution The elution rate was about 1 ml per 3 min The eluate was collected in 5 5 ml fractions and the collection started at the time when the material was put on the column

*Analyses on Eluted Material*

Each fraction was assayed for virus haemagglutinins To 0 2 ml samples was added 0 2 ml of a 0 4 per cent suspension of guinea pig erythrocytes and haemagglutination was carried out at 4° C The ultraviolet absorption of the individual fractions was estimated at 260 mμ and 280 mμ and the whole spectrum in the range 230 370 mμ was determined on selected fractions

The absorption at 430 mμ was used to follow the elution of phenol red which at this wavelength exhibits a pronounced absorption peak Phenol red also absorbs the ultraviolet light throughout the entire 230–370 mμ range The readings were therefore corrected by subtraction of the absorption due to the calculated amount of phenol red present in each fraction

## RESULTS

Experiments were made with sephadex which had been equilibrated with phosphate buffers of varying pH and molarity (Table 1) In the experiment with the 0 01 M, pH 7 2 buffer (Fig 1) the haemagglutinins were eluted in fractions 7–13 comprising a total volume of 38 5 ml compared with a volume of 25 ml for the material put on the column The recovery of haemagglutinins by the elution was 54 per cent The distribution of the agglutinins in the positive fractions gave no evidence for any separation into haemagglutinins with different affinities to the bed material and the ultraviolet absorption of the fractions had all a minium absorption at 251 252 mμ The 280/260 ratios were about 1 3 The absorption at 260 mμ and 280 mμ of the individual positive fractions followed closely the individual amounts of the haemagglutinins When compared with the positive fractions, the amount of ultraviolet absorbing material in the negative fraction 14 is at the limit for a positive haemagglutination test

The elution of virus haemagglutinins was followed by fractions 15 20 which contained very small amounts of ultraviolet absorbing material The subsequent fractions 21–40 contained material absorbing at 260 and 280 mμ and, in addition, fractions 28 40 contained phenol-red

Sodium chloride is the dominating part of Hanks balanced salt solution Its position in the elution diagram was qualitatively indicated by

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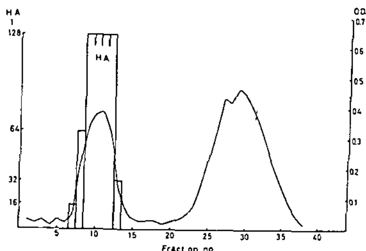



*Fig 1*

Gel filtration of SE polyoma virus haemagglutinin on Sephadex G-75
Polyoma virus in Eagle Hank with phenol red as indicator
——— U V material at 280 λ
– – – – *phenol red at 430 λ*
Elution with 0 01 M, pH 7 2 phosphate buffer

fractions which contained haemagglutinins The filtration also removed the phenol red indicator from the virus suspension On the other hand, no separation of virus haemagglutinins from high molecular non viral components was achieved, and the absorption spectrum of the isolated fractions was apparently dominated by the latter (E 280/260 = 1 3) A moderate dilution of the virus suspension occurs during the desalting procedure but a rapid concentration could again be achieved by treatment of the virus suspension with dry sephadex In our experiments the elution of the material has been made with phosphate buffers of varying pH and molarity, but in the conditions described no significant difference was found in the elution patterns The salt composition and the pH of the eluted virus fractions will correspond to those of the equilibration buffer Filtrations with sephadex as bed material is therefore convenient compared with conventional dialysis when it is desirable to change the suspending medium for a virus suspension The removal of small molecular substances and phenol red from the virus suspension will greatly facilitate analyses with which these materials interfere In many experiments a more or less pronounced loss in recovered HA units was found In some experiments, however, the recovery was equal to or exceeded the HA units put on the column It is therefore probable that a failure to obtain maximum recovery is most likely due to uncertainties in the HA determinations A small virus inactivation or loss due to other causes can however not be ruled out

## MATERIALS

The sera were obtained from pregnant women or blood donors presenting no demonstrable irregular blood type antibodies They contained no additives Most of these were tested either fresh or after storage at 4° C for up to 4 days a few were tested after storage at —22° C for up to 5 months

## METHODS

*Titration of the iso agglutinins* The sera were tested in saline using doubling dilutions Fresh red cells collected in ACD solution once washed and made up to a 0 25 per cent suspension were added in equal amounts The tubes were kept for 18 hours at 4° C followed by one hour at room temperature before they were examined for agglutination The titres were recorded as the reciprocal of the final dilution giving macroscopic agglutination Testing of the incomplete anti A and anti B This was performed by the indirect antiglobulin test after neutralization of the agglutinins as described by *Dunsford & Bowley* (3) A broadspectrum antiglobulin reagent was used The titres were recorded as the reciprocal of the final dilution giving agglutination by microscopic examination

*The complement fixation test* The complement source was fresh human serum or serum stored at —22° C It was from an AB Le(a b ) donor without irregular antibodies The guinea pig complement was either fresh or stored as mentioned above The complement was titrated in saline and tested against the same indicator system as in the actual test It was required that the complement containing serum should have an activity strong enough to be diluted 1 in 4 for human complement and 1 in 14 for guinea pig complement The dose of complement was 2 times the minimal dose giving a 100 per cent haemolysis

The pH of the serum saline dilutions was found to be about 7 4 a suitable val

red cells were of type Le(a b ) They were collected in ACD solution used fresh or stored at 4° C for up to two weeks and washed in saline at least 3 times before use The stored cells gave just as good results as the fresh ones The cells were used in a 10 per cent suspension in saline

*The*
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The
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0 3 ml
added
minute the supernatant was tested against 0 4 ml of the indicator system at 37° C for 15 min The supernatant showed as a rule no haemo lysis probably beca
fuged and the amour
was estimated by gr
small traces of haer
as the final dilution
Group O cells were a a negative control

## RESULTS

142 random sera from pregnant women were tested against group $A_1$, B and O human red cells by the complement fixation test and afterwards the ABO grouping was carried out as blind tests' The results are shown in Table 1

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# NATURALLY OCCURRING ANTI-A AND ANTI-B AND THEIR ABILITY TO FIX COMPLEMENT

*By*

PEDER ØSTGÅRD and HARALD ØRJASÆTER

Received 28.iv.62

Anti-A (and anti-B) antisera from different subjects may differ greatly in their serologic properties. They are usually divided into two classes: "natural" and "immune"; this division is convenient but not absolute. The classes have been differentiated by eight properties (7) of which the most "characteristic" are that "immune" antibodies haemolyse group $A_1$ (group B) human red cells, sensitise red cells to the antiglobulin test even after neutralization with an excess of AB substance, have an optimum temperature of 37° C, react with pig A-antigen, and fix complement – whereas "natural" antibodies do nothing of this.

Hitherto few investigators have studied the ability to fix complement. To the best of our knowledge the first investigation was carried out by *Schiff & Adelsberger* in 1924 (9); "normal" human sera were tested and only 3 out of more than 100 sera were found to fix complement when tested against human red cells of group A, B or AB. Later, complement fixing antibodies with A-specificity were found by *Witebsky* (10) in a group O mother who was delivered of a child suffering from a haemolytic disease. This author reported that the "naturally" occurring isoagglutinins did not present this ability of fixing complement.

*Ervin et al.* (4, 5) demonstrated a presence of complement fixing antibodies with A-specificity in sera from "dangerous" universal donors. None of these had ever received transfusion or other known stimuli for a development of erythrocyte antibodies of immune type; nevertheless the sera appeared to have several of the characteristics attached to the immune antibodies.

This work deals with further investigations on the complement fixing ability of the iso antibodies of the ABO-system.

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The authors are greatly indebted to *Dr. George Discombe* of London for reading the manuscript and making valuable suggestions.

TABLE 2

*Comparison of the Complement Fixation Titres and the Titres by Antiglobulin Test after Partial Neutralization*

| Serum number — Group O | Complement fixation test | | | | | | | Indirect antiglobulin test after partial neutralization | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 |
| 831* | ++++ | ++++ | ++++ | ++ | + | | | — | | | | | | | |
| 004 | ++++ | ++++ | ++++ | +++ | + | — | | — | | | | | | | |
| 992 | ++++ | ++++ | +++ | + | | | | — | | | | | | | |
| 939 | ++++ | ++++ | ++++ | ++ | + | - | | — | | | | | | | |
| 023 | ++++ | ++++ | ++++ | +++ | + | | | — | | | | | | | |
| 892 | ++++ | ++++ | ++++ | +++ | ++ | | | — | | | | | | | |
| 017 | ++++ | ++++ | ++++ | ++ | + | — | | + | — | | | | | | |
| 835 | ++++ | ++++ | ++++ | ++++ | + | | | + | — | | | | | | |
| 766 | ++++ | ++++ | ++++ | ++ | — | | | — | | | | | | | |
| 624 | ++++ | ++++ | +++ | ++ | + | | | — | | | | | | | |
| La † | ++++ | ++++ | ++++ | ++++ | +++ | ++ | — | +++ | +++ | +++ | +++ | ++ | + | (+) | — |
| Mo ** | ++++ | ++++ | ++++ | ++++ | +++ | ++ | — | — | | | | | | | |
| Ha § | ++++ | ++++ | ++++ | ++++ | +++ | ++ | — | + | (+) | — | | | | | |
| Fn § | ++++ | ++++ | +++ | ++ | — | | | ++ | +(+) | + | (+) | — | | | |
| Bf // | ++++ | ++++ | ++++ | +++ | ++ | | | ++ | ++ | ++ | +(+) | + | + | — | |
| Be // | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | — | + | + | (+) | (+) | — | | | |
| Vo • a) | ++++ | ++++ | +++ | — | | | | +++ | +++ | ++ | + | + | (+) | — | |
| b) | +++ | — | | | | | | ++ | + | + | (+) | — | | | |

a) Against group A human red cells b) Against group B human red cells

* Ten selected sera from pregnant women of group O with positive complement fixation test but without any indications of ABO-erythroblastosis of the foetus

† Sample taken 3 months after delivery of a child with ABO erythroblastosis. Exchange transfusion

** Sample taken 10 days after delivery of a child, vague clinical indications of ABO erythroblastosis

§ Samples taken in the 7th month of pregnancy, para III, the second child was born with clinical indications of ABO erythroblastosis. Exchange transfusions

// Samples taken at delivery. Clinical indications of ABO erythroblastosis. Exchange transfusions

• Sample taken 3 month after delivery, para II, clinical indications of ABO-erythroblastosis (Father and the second child of group A)

TABLE 1

*Testing of 142 Random Sera from Pregnant Women by the Complement Fixation test, with the Antigens* $A_1$*, B and O (Performed as "Blind Tests")*

| Serum from group | Number investigated | Antigens | | | | | | | | | | | | | Read as pos ≧ ++ | Read as neg ≦ + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $A_1$ | | | | | B | | | | | O | | | | |
| | | − | + | ++ | +++ | ++++ | − | + | ++ | +++ | ++++ | − | + | ++ | | |
| A | 71 | 71 | 0 | 0 | 0 | 0 | 48 | 4 | 2 | 4 | 13 | 71 | 0 | 0 | 19 | 52 |
| B | 8 | 3 | 0 | 0 | 1 | 4 | 8 | 0 | 0 | 0 | 0 | 8 | 0 | 0 | 5 | 3 |
| AB | 4 | 4 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 4 |
| O | 59 | 15 | 2 | 4 | 3 | 35 | 39 | 6 | 0 | 4 | 10 | 58 | 1 | 0 | 13* | 16† |

− complete haemolysis,
\+ about 25 per cent haemolysis,
++ about 50 per cent haemolysis,
+++ about 75 per cent haemolysis,
++++ 100 per cent haemolysis
* Positive against $A_1$ and B antigen
† Seventeen negatives against $A_1$ antigen one of these being positive against B, which makes sixteen as shown in the table

The correlation is shown in Fig 1 presenting also the 7 sera from mothers delivered of babies suspected of having haemolytic diseases On the whole, sera without demonstrable complement fixing activity had an agglutinin titre below 1/128–1/256 On the other hand, all sera with complement fixing ability had agglutinin titres above 1/64 and most of these above 1/128 Moreover, the titres of complement fixing anti bodies were approximately proportional to the agglutinin titres The 7 pathological sera showed no deviations from the rule Most of these had comparatively high titres, agglutinin titres as well as complement fixation titres, but they were as scattered as were the 'normal' ones in Fig 1

TABLE 3

*Comparison of Human and Guinea Pig Complement in the Complement Fixation Test The Tests are Performed against Group $A_1$ Human Red Cells in Group O Sera from Pregnant Women*

| | | Guinea pig complement | | Sum |
|---|---|---|---|---|
| | | + | — | |
| Human complement | + | 6 | 37 | 43 |
| Human complement | — | 0 | 11 | 11 |
| Sum | | 6 | 48 | 54 |

All sera positive with guinea pig complement gave higher titres with human complement

It was thought that the source of complement in some way or other might influence the sensitivity of the complement fixation test To investigate this we performed parallel complement fixation tests both with human and with guinea pig complement under identical conditions (Table 3) The table shows a significantly higher frequency of positive results obtained with human complement than with guinea pig complement The titres also were higher using human complement The importance of different antigens was more closely examined by testing in parallel some anti A containing group O sera against group $A_1$ and $A_2$ human red cells $A_2$ cells were regularly found to give lower titres than $A_1$ cells most frequently the difference was found to be about 2 titre steps Similarly, by testing group O sera against $A_1$ and B cells lower titres were generally found against B cells than against $A_1$ cells It is well known that the same is seen in agglutination tests

## DISCUSSION

Preliminary tests were made with a view to making our complement fixation test as sensitive as possible, and special precautions were taken to avoid non specific reactions The amount of complement and the degree of concentration of the red cells were both as low as they could

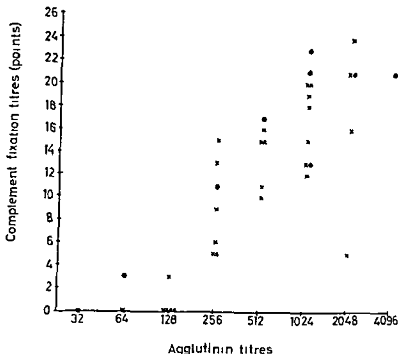



*Fig 1*

*Correlation between the complement fixation titres against group A and B human* red cells and the agglutinin titres against the same red cells

The complement fixation titres recorded as points ++++ = 4 points
+++ = 3 points
++ = 2 points
\+ = 1 points
— = 0 points

× sera from blood donors
● the last 7 sera shown in table 2

The findings show that the complement fixing ability has the same specificity as the iso-agglutinins in the ABO-system, and that more than two thirds of the tested anti A sera and about one fourth of the anti-B sera bind complement Only in one serum a weak positive reaction occurred against the O(H) antigen used as control This indicated *that non-specific fixation of complement had not influenced our results* Thus, the frequency of complement fixing antibodies was found to be unexpectedly high as compared with the earlier observations Seventeen of the positive group O sera were therefore examined with the indirect antiglobulin test after neutralization of agglutinins as mentioned before (Table 2) Of these, 10 sera were from unselected "normal" pregnant women, while 7 sera were from mothers delivered of group A or B babies suspected of having haemolytic diseases due to anti-A or anti-B In the "normal" sera no or only doubtful traces of incomplete anti A or anti-B were *observed, although complement fixing antibodies with* relatively high titres could be demonstrated

Further, 29 sera from blood donors were tested an[illegible] complement fixing antibodies were compared with the ag[illegible]

explained, but similar observations have been reported comparing the haemolysing ability with that of the agglutination (1) Thus, the problem seems rather complex, although it is justifiable to conclude that the property of fixing complement is not a special characteristic of the "immune" iso-antibodies anti-A or anti-B, as has been usually supposed, but rather an expression of the strength of the agglutinins, possibly with an addition of complement fixing incomplete anti-A or anti B antibodies That a high agglutinin titre by itself to some extent may be an expression of immunisation is another question The difference between the so called "immune" and "natural" anti-A or anti-B is probably best regarded as a quantitative difference, as has been emphasized also by *Mollison* (6) who pointed out that probably all anti-A sera contained at least traces of "immune" anti-A It is, however, improbable that the complement fixing activity found should be dependent on traces of "immune" antibodies

## SUMMARY


This investigation deals with the complement fixing ability of anti A and anti B antibodies as shown by a complement fixation test

Of 142 random sera from pregnant women more than two thirds were found to fix complement with $A_1$ human red cells and about one fourth with B cells All complement fixing sera showed agglutinin titres higher than about 1 128 and an approximate proportionality between the agglutinin titre and the complement fixation titre could be demonstrated

The titration of complement fixing antibodies against different antigens such as $A_1$, $A_2$ and B human red cells, showed the same correlations as are well known for the agglutinin titres

Ten group O sera from pregnant women showing relatively high complement fixation titres but otherwise unselected, were examined by an antiglobulin test after partial neutralization No incomplete anti-A or anti B antibodies were found On the other hand, 7 sera from mothers presenting clinical evidence of ABO-immunization, most of these showing incomplete anti A or anti B antibodies, gave about the same complement fixation titres as the 10 sera mentioned above

The complement fixation test was found considerably more sens[illegible]

[illegible] character of the anti-A or anti-B antibodies


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be without making reading difficult We believe that this is one reason for the high frequency of positive results found On the other hand, the experiments performed as "blind tests" confirms the specificity of our reactions (Table 1)

Table 1, in which one + reaction is read as *negative*, shows that the complement fixing antibodies have the same specificity as the agglutinins anti-A or anti-B It is therefore highly improbable that the high frequency of complement fixing antibodies found is due to non-specific reactions, even though the results clearly differ from the investigations mentioned in the introduction

*Schiff & Adelsberger* as well as *Witebsky* used guinea pig complement We also found a considerably lower frequency of positive reactions with guinea pig complement than with human complement Moreover, Witebsky in his experiments used AB-substance from amniotic fluids as an antigen (10)

In the sera from "normal" pregnant women we found that more than two thirds of the anti-A and about one fourth of the anti-B antibodies fixed complement Although a certain amount of scattering exists, the titres of the complement fixing antibodies are approximately proportional to those of the agglutinins having a titre higher than about 1/128 The titres were regularly found to be lower against $A_2$ cells than against $A_1$ cells and lower against B cells than against $A_1$ cells in group O sera as is usual in agglutination tests All of these findings indicate that the *naturally occurring anti-A or anti-B antibodies fix complement* if their agglutinin titres are sufficiently high Evidence of this is also present in the observations of *Dacie* (2) demonstrating the adsorption of complement to "non-immune" types of anti-A or anti-B antibodies, also found to be of the "non-gammaglobulin" type That naturally occurring anti-A may consist of "non-gammaglobulin" antibodies is also indicated by the findings of *Renton* (8) The antibodies of the "non-gammaglobulin type are usually regarded as complement fixing antibodies

*The complement fixing property has formerly been regarded* as a characteristic of the "immune" iso-antibodies, in the same way as demonstration of incomplete anti-A or anti-B by the indirect antiglobulin test after partial neutralization This can not be confirmed by our experiments In our investigation 7 sera were included in which incomplete anti-A or anti-B were found in most of the cases These sera did not show complement fixation titres of unusual height, but they were very similar to the titres of the "normal" sera included in Table 2 and Fig 1 The content of incomplete antibody seems not to have noticeably influenced the complement fixing ability, but a certain influence cannot be precluded, because the titres of incomplete antibodies were low as compared with the agglutinins

In Fig 1 one serum is noted which clearly differs from the others it had a high agglutinin titre but showed a low complement fixation titre, also confirmed by re-testings This discrepancy cannot be fully

FROM THE BACTERIOLOGICAL DEPARTMENT OF SERAFIMERLASARETTET
STOCKHOLM (HEAD ARNE LITHANDER M.D.)

# THE PASSAGE OF PENICILLIN INTO THE CEREBROSPINAL FLUID AFTER PARENTERAL ADMINISTRATION IN STAPHYLOCOCCIC MENINGITIS

## I. *An Experimental Investigation on Rabbits*

*By*

ARNE LITHANDER and BRITA LITHANDER

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One of the requirements of antibiotic treatment of infections is that the substance used be present in sufficiently high concentration in the center of infection. This requirement is sometimes difficult to satisfy, particularly in cases of infection in the cerebral meninges and in the brain. We have clinical experience of the passage of different substances to the cerebrospinal fluid and the brain substance in these conditions in man, but experimental investigations in the field are lacking.

The fact that even with high concentrations in the blood the majority of chemotherapeutic preparations penetrate only poorly into the cerebrospinal fluid and the brain when the cerebral meninges are intact is believed to be due to the blood cerebrospinal fluid barrier and the blood brain barrier.

It has been learned from investigations on healthy subjects that if the meninges are intact there is very little if any permeation of the cerebrospinal fluid following parenteral administrations of moderate doses of the majority of antibiotics. The blood cerebrospinal barrier is nevertheless not entirely impermeable. This is evident, for example, from the fact that even when the meninges are intact penicillin can be detected in the cerebrospinal fluid after very large doses. However, the concentration of penicillin in the cerebrospinal fluid is very low even after large doses. *Boger, Baker & Wilson* (1) and *Goodman & Gilman* (2) found that the concentration of penicillin in the plasma generally was 10 units per millimeter or more in cases in which penicillin was present in the cerebrospinal fluid after parenteral administration to persons with intact meninges. When the meninges were inflamed, on the other hand, the effectiveness of the blood cerebrospinal fluid barrier

The penicillin in the present investigation was kindly supplied by *AB KABI*, Stockholm, Sweden.

Potentially

3 D 152 Oliver
and Boyd Edinburgh
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7 *Reepmaker J & van Loghem J J Jr* Anti A and anti B immune antibodies in pregnancy Clinical date of 240 families Vox Sang *3* 143 161 1953
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animals and immature ones as regards the functional capacity of the blood-cerebrospinal fluid and the blood brain barrier *Bakay* (12), for example stated that the passage of radioactive phosphorus into the cerebrospinal fluid was more rapid in young than in adult rabbits Other workers have attained the same results in principle, using trypan blue *Grontoft* (13), on the other hand, could find no difference in this respect between newborn and adult rabbits in experiments with trypan blue

The present investigation represents an attempt to complete clinical observations made hithertho by studies of the passage of various antibiotica across the blood cerebrospinal barrier in infections of the central nervous system in animals For various reasons it was considered advisable to begin with penicillin

## MATERIALS AND METHODS

Adult rabbits weighing between 2 and 3 kilograms were used for the most part Young rabbits between the ages of three and four months weighing 1 4 and 1 5 kg were used in one series The backs of the necks were shaved and the animals were given intravenous anaesthesia consisting of 0 4 ml of 6 per cent nembutal per kilogram The cisterna magna was punctured and 0 5 ml of cerebrospinal fluid was withdrawn from the grown animals and 0 2 ml from the young Immediately thereafter

animals and the young ones were given intracisternal injections of 0 5 ml and 0 2 ml respectively of physiological saline solution The following day the rabbits were given injections of benzyl penicillin (penicillin G)[1] when they exhibited symptoms of meningitis which usually was the case 20 to 22 hours after the injection of staphylococci The controls and the experimental animals received the injections by pairs The control rabbits were given injections of the same amount of penicillin and after the same interval as the corresponding experimental animals The injections of penicillin were administered intravenously The dosage was 20 000 units

... 30 minutes after the injection of penicillin The cerebrospinal fluid was removed with the utmost care in order

... four minutes after the beginning of the cisternal puncture The blood was immediately centrifuged and the plasma sepa-rated The ...

... of the brain were put into formalin for the pathological examination The greater part of the brain was saved for penicillin determination In this connection the lateral cerebral ventricles were slit Blood and cerebrospinal

[1] Hereinafter the term "benzyl penicillin" will be abbreviated to "penicillin"

appeared to decrease and antibiotics passed more easily into the fluid At the same time, *Kelentei & Foldes* (3) *reported that in cases of con*tinous intravenous administration a concentration of 5 to 10 units of penicillin per millimeter of blood plasma is needed to achieve therapeutically effective concentrations in the cerebrospinal fluid Most workers consider, however, that in meningitis a suitable therapeutic concentration can be produced in the cerebrospinal fluid with a lower concentration in the plasma also

*Friederiszick & Hoffecher* (4) investigated the relationship between the concentration of penicillin in plasma and in cerebrospinal fluid in children with meningococcic meningitis and pneumococcic meningitis They found the ratio to be 7–57 1 in the acute stage of the disease and 23 *100 1 when the patients had almost completely recovered In studies* with fluorescein in adult cases of meningococcic meningitis, *Lange, Schwimmer & Boyd* (5) *noted a mean ratio of 16 1 between the con*centration in plasma and in cerebrospinal fluid in the acute stage – The picture *is different in other types of infection For example, Baumann & Kardos* (6) found that inveterate neurosyphilis did not affect the blood-cerebrospinal fluid barrier in such a way that penicillin permeated the cerebrospinal fluid *McIlwain* (7) reported that even if a dose as large as 25 million units of penicillin was introduced intravenously in neurosyphilis so that the plasma concentration exceeded 50 units per millimeter, the concentration in the cerebrospinal fluid was at most 0 5 units per millimeter *Krautwald & Kolmar* (8) assumed that the permeability of a substance may be proportional to the extent of the inflammatory process in the meninges *Boger & Wilson* (9) reported that the penetration of penicillin into cerebrospinal fluid apparently is *not parallel to the intensity of the inflammation, if the latter is judged* by the degree of pleocytosis

*Experimental studies* on animals have revealed that, after intravenous injections of chemotherapeutic preparations, the permeation through the nervous system is much slower and less pronounced than *permeation through other parts of the organism In experiments* on healthy, adult rabbits, *Foldes & Kelentei* (10) found that no penicillin penetrated the cerebrospinal fluid following intravenous administration of doses of 2,000 to 15,000 units per kilogram No differences could be found between *anaesthetized and non-anaesthetized animals In ex*periments on healthy animals, other workers have observed that enor*mous intravenous or intramuscular doses of penicillin are required if* penicillin is to be found in the cerebrospinal fluid

Few experiments have been made on animals with infections in the central nervous system *Finck* (11) found that the permeability of the blood-cerebrospinal fluid barrier by $P^{32}$ was much greater in adult rabbits in which meningitis had been produced by intrathecal injection of streptococci and staphylococci than in healthy animals

Some investigatons *appear to have revealed differences between adult*

in the majority of the cases The control rabbits exhibited no signs of meningitis or other conditions and their cerebrospinal fluid was clear

**TABLE 1**

*Penicillin Concentrations in Plasma Cerebrospinal Fluid and Brain*

| Group | Plasma (P) | Cerebrospinal Fluid (F) | Brain (B) | % Quotient F/P | B/P |
|---|---|---|---|---|---|
| | *Adult rabbits 5 000 I U penicillin kg* | | | | |
| Controls | 1.61 ± 0.18 | 0.30 ± 0.00 | 0.59 ± 0.06 | 5 | 10 |
| | 0.41 (5) | (4) | (3) | | |
| Meningitis all degrees | 1.58 ± 0.10 | 0.92 ± 0.03 | 0.72 ± 0.07 | 22 | 14 |
| | 0.25 (6) | 0.22 (6) | 0.16 (5) | | |
| | *Adult rabbits 20 000 I U penicillin kg* | | | | |
| Controls | 2.33 ± 0.05 | 0.68 ± 0.04 | 0.83 ± 0.04 | 2 | 3 |
| | 0.28 (32) | 0.20 (32) | 0.24 (32) | | |
| Meningitis | | | | | |
| + | 2.36 ± 0.13 | 1.20 ± 0.12 | 1.01 ± 0.09 | 7 | 4 |
| | 0.29 (5) | 0.26 (5) | 0.20 (5) | | |
| ++ | 2.21 ± 0.15 | 1.27 ± 0.07 | 1.01 ± 0.08 | 11 | 6 |
| | 0.39 (7) | 0.19 (7) | 0.20 (7) | | |
| +++ | 2.29 ± 0.06 | 1.57 ± 0.06 | 1.12 ± 0.05 | 19 | 7 |
| | 0.26 (20) | 0.25 (20) | 0.24 (20) | | |
| | *Young rabbits 20 000 I U penicillin kg* | | | | |
| Controls | 2.59 ± 0.06 | 0.90 ± 0.06 | 1.04 ± 0.04 | 2 | 3 |
| | 0.18 (10) | 0.18 (10) | 0.14 (10) | | |
| Meningitis | | | | | |
| + | 2.59 ± 0.16 | 1.26 ± 0.37 | 1.11 ± 0.14 | 5 | 3 |
| | (4) | (4) | (4) | | |
| + | 2.55 ± 0.09 | 1.52 ± 0.16 | 1.20 ± 0.88 | 9 | 4 |
| | (3) | (3) | (3) | | |
| +++ | 2.67 ± 0.14 | 2.11 ± 0.05 | 1.37 ± 0.17 | 28 | 5 |
| | (4) | (4) | (4) | | |
| | *Adult rabbits 80 000 I U penicillin kg* | | | | |
| Controls | 3.43 ± 0.16 | 1.23 ± 0.05 | 1.47 ± 0.09 | 1 | 1 |
| | 0.37 (5) | 0.12 (5) | 0.21 (5) | | |
| Meningitis all degrees | 3.38 ± 0.06 | 2.28 ± 0.16 | 1.49 ± 0.11 | | |
| | 0.15 (6) | 0.38 (6) | 0.26 (6) | | |

Penicillin concentrations expressed as log [100(number of penicillin units per ml)]

Each group of figures represents
mean ± error of mean
stand deviat (number of cases)

The quotients in the right hand column are calculated from the group means

The concentration of penicillin in the plasma rose (Table 1) as the intravenous penicillin doses were increased For example, the concentration was lowest using a penicillin dose of 5 000 units per kilogram and was on an average five times greater when the dose was 20 000 units per kilogram A dose of 80,000 units per kilogram resulted in approximately twelve times the concentration of a dose of 20,000 units per kilogram In each dosage series the concentration of penicillin in

fluid were washed from the brain with physiological saline solution after which the brain was dried with cellulose waste A sample of the brain was weighed and ground with sterile sand in a mortar The ground brain substance was carefully mixed with a fixed amount of buffer solution The supernatant fluid was removed after the brain suspension had been centrifuged

The samples of cerebrospinal fluid plasma and brain substance were collected at such short intervals that they may be said to have been taken practically simultaneously

The penicillin concentration of the cerebrospinal fluid the plasma and the brain extract was determined immediately after the samples were obtained

Diffusion technique in agar was used for determination of the penicillin content of plasma cerebrospinal fluid and brain substance A modification of the method described by *Klein* in 1957 (14) was applied using Subtilis spores of the ATCC 6633 strain as test organism The spores were suspended in melted agar in a concentration of 7 500 spores per millimeter of agar The agar was poured into petri dishes with plane bottoms which were placed on a horizontal base When the agar had solidified six or seven cylindrical holes nine milimeters in diameter were punched out on each plate 0 06 milliliters of phosphate buffer solutions of standard penicillin containing 0 12 0 06 and 0 03 units per milliliter were introduced into three of these holes in each plate The remaining holes were filled with 0 06 milliliters of the solutions of plasma cerebrospinal fluid or cerebral substance the penicillin content of which was to be determined An average of five plates was used in each test Readings were made following incubation at 37° C for 18 hours Two diameters at right angles of the inhibition zone were measured in a magnifying apparatus Only circular zones were approved A linear regression line between penicillin concentrations (log units) and diameters was calculated for the standard in each plate With the aid of this line the penicillin concentrations corresponding to the diameters obtained in the experimental animals were read

The error of the method described in the foregoing was detected by dissolving penicillin in fixed concentrations in the same buffer solutions as the one used in the determination of penicillin concentration in plasma cerebrospinal fluid and brain substance The investigation showed that the error of the method varied with the penicillin concentration in the buffer solution For determination of the concentration of penicillin in the different substances in the present experiments in rabbits we used concentrations in which the error of the method did not exceed 5 per cent

Only the penicillin not bound by plasma cerebrospinal fluid and brain substance could be determined in the experiments in rabbits described above The ability of these substances to bind penicillin was studied It was found that rabbit plasma in undiluted or only slightly diluted form had a great capacity of binding penicillin while cerebrospinal fluid and brain substance were less potent in this respect Dilution of these substances was followed by a decrease in their ability to bind penicillin The results of these studies and of the investigation of the error of the method will be published later

## RESULTS

All of the rabbits which were given injections of staphylococci in the posterior cistern exhibited signs of meningitis the day after the injection One group of rabbits which showed only mild signs of meningitis with some degree of opisthotonous and spasticity was labelled + Another group of rabbits with moderate opisthotonous and spasticity was labeled + + The majority of the infected rabbits *i e* 20 animals revealed pronounced signs with a high degree of opisthotonous and spasticity, as well as cramps which were spontaneous in some cases and in others occurred on provocation This group was labeled + + + The general condition in the different groups was affected to an extent corresponding to meningitis The cerebrospinal fluid was very cloudy

in the majority of the cases The control rabbits exhibited no signs of meningitis or other conditions and their cerebrospinal fluid was clear

TABLE 1

*Penicillin Concentrations in Plasma Cerebrospinal Fluid and Brain*

| Group | Plasma (P) | Cerebrospinal Fluid (F) | Brain (B) | % Quotient | |
|---|---|---|---|---|---|
| | | | | F P | B I |
| | *Adult rabbits 5 000 I U penicillin kg* | | | | |
| Controls | 1 61 ± 0 18<br>0 41 (5) | 0 30 ± 0 00<br>(4) | 0 59 ± 0 06<br>(3) | 5 | 10 |
| Meningitis all degrees | 1 58 ± 0 10<br>0 25 (6) | 0 92 ± 0 09<br>0 22 (6) | 0 72 ± 0 07<br>0 16 (5) | 22 | 14 |
| | *Adult rabbits 20 000 I U penicillin kg* | | | | |
| Controls | 2 33 ± 0 05<br>0 28 (32) | 0 68 ± 0 04<br>0 20 (32) | 0 83 ± 0 04<br>0 24 (32) | 2 | 3 |
| Meningitis | | | | | |
| + | 2 36 ± 0 13<br>0 29 (5) | 1 20 ± 0 12<br>0 26 (5) | 1 01 ± 0 09<br>0 20 (5) | 7 | 4 |
| ++ | 2 21 ± 0 15<br>0 39 (7) | 1 27 ± 0 07<br>0 19 (7) | 1 01 ± 0 08<br>0 20 (7) | 11 | 6 |
| +++ | 2 29 ± 0 06<br>0 26 (20) | 1 57 ± 0 06<br>0 25 (20) | 1 12 ± 0 05<br>0 24 (20) | 19 | 7 |
| | *Young rabbits 20 000 I U penicillin kg* | | | | |
| Controls | 2 59 ± 0 06<br>0 18 (10) | 0 90 ± 0 06<br>0 18 (10) | 1 04 ± 0 04<br>0 14 (10) | 2 | 3 |
| Meningitis | | | | | |
| + | 2 59 ± 0 16<br>(4) | 1 26 ± 0 37<br>(4) | 1 11 ± 0 14<br>(4) | 5 | 3 |
| ++ | 2 55 ± 0 09<br>(3) | 1 52 ± 0 16<br>(3) | 1 20 ± 0 88<br>(3) | 9 | 4 |
| +++ | 2 67 ± 0 14<br>(4) | 2 11 ± 0 05<br>(4) | 1 37 ± 0 17<br>(4) | 28 | 5 |
| | *Adult rabbits 80 000 I U penicillin kg* | | | | |
| Controls | 3 43 ± 0 16<br>0 37 (5) | 1 23 ± 0 05<br>0 12 (5) | 1 47 ± 0 09<br>0 21 (5) | 1 | 1 |
| Meningitis all degrees | 3 38 ± 0 06<br>0 15 (6) | 2 28 ± 0 16<br>0.38 (6) | 1 79 ± 0 11<br>0.26 (6) | | |

Penicillin concentrations expressed as log [100(number of penicillin units per ml)]

Each group of figures represents

mean ± error of mean

stand deviat (number of cases)

The quotients in the right hand column are calculated from the group means

The concentration of penicillin in the plasma rose (Table 1) as the intravenous penicillin doses were increased For example, the con centration was lowest using a penicillin dose of 5 000 units per kilogram and was on an average five times greater when the dose was 20 000 units per kilogram A dose of 80 000 units per kilogram resulted in approximately twelve times the concentration of a dose of 20 000 units per kilogram In each dosage series the concentration of penicillin in

the plasma was approximately the same in the experimental animals as in the controls The dose of 20,000 units per kilogram gave a somewhat higher average plasma concentration in young animals than in adult rabbits

Table 1 shows that using a penicillin dose of 20,000 units per kilogram the penicillin penetration from plasma into brain tissue and cerebrospinal fluid increased with the degree of infection (O, +, ++, +++) This is best illustrated by the trend of the quotient values Adult and young rabbits did not differ much in this respect, nor did the groups with penicillin doses of 5,000 and 80,000 units The percentual penetration into brain tissue and cerebrospinal fluid was best with low penicillin dosage (5,000 units), and appeared to decrease with increasing plasma levels

Most of the groups are relatively small It is clear, however, that the penicillin contents of cerebrospinal fluid and brain were significantly larger in animals with meningitis than in the controls

At the same time, the use of quotients to compare penetrations is not entirely satisfactory Instead, the direct correlation between cerebrospinal fluid, brain and plasma values, as well as differences between such correlations, should be examined Multiple correlations were also calculated

Three correlations *viz* cerebrospinal fluid-plasma (Fig 1, 5), brain plasma (Fig 2, 6), and cerebrospinal fluid-brain (Fig 3) as well as the relation between the levels of penicillin and blood corpuscle (white, Fig 4) in the cerebrospinal fluid, were studied and plotted in diagrams Owing to the pronounced skewness in the statistical distributions of both penicillin values and corpuscles, logarithmic values were used throughout, in diagrams and in calculations The three first-mentioned correlations were plotted graphically for young and adult animals, for the three penicillin doses, and for all degrees of meningitis (O, +, ++ +++) separately (Fig 1, 5, 2, 6, 3) Clear correlations were observed and regressions were calculated for the largest group (penicillin dose of 20,000 units in adults) and separately for the various degrees of meningitis (Fig 1, 2, 3) The regression lines are indicated in the diagrams For the other groups all values were plotted and the regression line for the corresponding adult group which received 20,000 unit doses was included for comparison (Fig 5 and 6)

The correlations are indicated in Table 2

The correlations are clearly significant in the larger groups and numerically high in the other cases Since we have here three variables which have been interrelated it would be interesting to determine whether they are all true relationships Multiple correlation coefficients were therefore also calculated In principle this means the removal of the influence of one factor upon the correlation between the other two The result appears in Table 3 This was done for the two larger groups controls and cases of meningitis +++

TABLE 2

*Correlations between Penicillin Contents in the Various Media*

| Degree of meningitis | y = cerebrospinal fluid penicillin<br>x = plasma penicillin | y = brain penicillin<br>x = plasma penicillin | y = cerebrospinal fluid penicillin<br>x = brain penicillin |
|---|---|---|---|
| Controls (32 cases) | +0.59<br>y = 1.76 + 0.84 x<br>0.23 | +0.82<br>y = 1.54 + 0.95 x<br>0.16 | +0.48<br>y = 0.43 + 0.59 x<br>0.22 |
| Meningitis + (5 cases) | +0.95<br>y = 1.10 + 1.05 x<br>0.11 | +0.94<br>y = 1.00 + 1.34 x<br>0.11 | +0.94<br>y = 0.14 + 0.73 x<br>0.08 |
| Meningitis ++ (7 cases) | +0.71<br>y = 0.30 + 1.50 x<br>0.30 | +0.80<br>y = 0.62 + 1.56 x<br>0.26 | +0.72<br>y = 0.03 + 0.77 x<br>0.13 |
| Meningitis +++ (20 cases) | +0.82<br>y = 0.99 + 0.83 x<br>0.15 | +0.77<br>y = 1.38 + 0.82 x<br>0.17 | +0.69<br>y = 0.09 + 0.65 x<br>0.18 |

Penicillin dose 20 000 units per kg. Penicillin concentrations expressed as log [(100 number of penicillin units per ml)]

Each group of figures represents

corr coeff
*regression line*
stand dev
around
regr line

Statistically significant correlation coefficients are *underlined* ($P < 0.01$)

TABLE 3

*Multiple Correlations between Plasma, Brain and Cerebrospinal Fluid Penicillin Concentrations Corresponding to the Direct Correlations in Table 2. Adult Rabbits*

| Direct correlations | Multiple correlations |
|---|---|
| Controls (32 cases) | |
| r(F/P) = +0.59 | r(F/P.B) = +0.40 |
| r(B/P) = +0.82 | r(B/P.F) = +0.76 |
| r(F/B) = +0.48 | r(F/B.P) = +0.01 |
| Meningitis +++ (20 cases) | |
| r(F/P) = +0.82 | r(B/P.F) = +0.63 |
| r(B/P) = +0.77 | r(F/B.P) = +0.49 |
| r(F/B) = +0.69 | r(F/P.B) = +0.42 |

Penicillin dose 20 000 units per kg. Correlation coefficients (r). Plasma (P), cerebrospinal fluid (F), brain (B). Significant correlation coefficients ($P < 0.01$) are *underlined*

It will be seen from Table 3 that only the correlations of cerebrospinal fluid and brain to plasma are true, whereas there is no true relationship between cerebrospinal fluid and brain concentrations. The direct correlation observed between the latter two would thus be only a secondary effect derived from the fact that both of these two factors are correlated to a third, the plasma values. Thus the penicillin content

Correlation between cerebrospinal fluid (CSF) and *plasma concentrations* of penicillin at various degrees of meningitis Adult rabbits receiving 20 000 I U pc
Regression lines pooled
○ controls
▲ meningitis 1st degree
△ 2nd
● 3rd

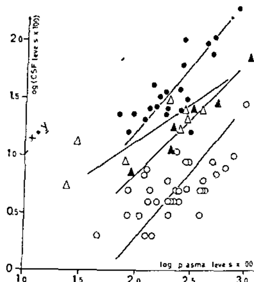



*Fig 1*

Correlation between *brain and plasma* concentrations of penicillin at various *degrees of meningitis* Adult rabbits receiving 20 000 I U pc
Regression lines pooled
○ controls
▲ meningitis 1st degree
△ 2nd
● 3rd

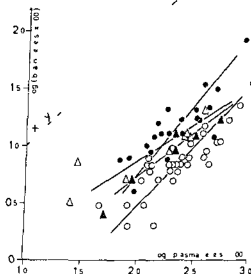



*Fig 2*

of the cerebrospinal fluid seems to be wholly controlled by the plasma level This also applies to the penicillin concentrations of the brain Any exchange of penicillin between brain and cerebrospinal fluid, however, is probably purely random

*The penicillin concentration in the cerebrospinal fluid was consistently higher in rabbits with meningitis than in the control rabbits without meningitis* (Table 1 and Fig 1) This was the case regardless of the size of the dose of penicillin and of the age of the rabbits In the series in which the dose of penicillin was 20 000 units per kilogram, the concentration was approximately 5, in the series given doses of 80,000

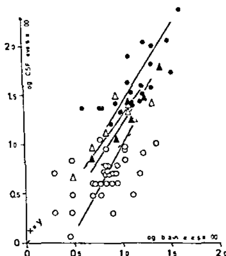



*Fig 3*

units it was approximately 11 and with a dosage of 5 000 it was approximately four times as high in the experimental rabbits animals as in the corresponding controls (Table 1)

Figure 1 shows the relation between the penicillin concentration in the cerebrospinal fluid and the penicillin concentration in the plasma at different degrees of the clinical signs of meningitis in adult experimental animals given doses of 20 000 units of penicillin per kilogram and in control animals who received the same dose of penicillin

Table 1 and Figure 1 reveal that the concentration of penicillin in all rabbits was higher in plasma than in cerebrospinal fluid

*At a certain plasma concentration the cerebrospinal fluid concentration increased with the intensity of the signs of meningitis* Within a specific clinical picture the concentration in the cerebrospinal fluid invariably appeared to increase with the plasma concentration This appears to be the case in all degrees of meningitis *i e* from Group O comprising the control animals to Group + + + If the concentration in the cerebrospinal fluid in clinical groups + and + + + is compared with the concentration in the control group the quotient between them is constant in each clinical group (constant log distance) regardless of the plasma concentration at which the comparison is made Group + + appears to deviate somewhat in this respect probably because this intermediate group is more difficult to define than groups + and + + +

*The penicillin concentration in the brain was consistently higher in rabbits with meningitis than in controls (Table 1 and Fig 2)* This was the case regardless of the size of the dose of penicillin and of the age of the experimental animals (Fig 6)

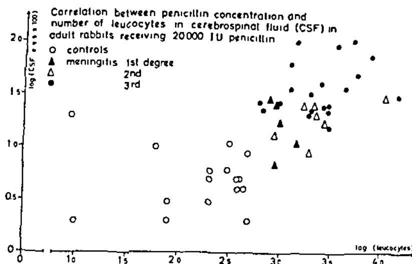



*Fig 4*

Table 1 and Figure 2 reveal that the concentration of penicillin in all rabbits was higher in plasma than in brain substance An increase of the plasma concentration was accompanied by an increase in the penicillin concentration in the brain, but the increase was relatively independent of the degree of meningitis

The relations between the concentration of penicillin in the cerebrospinal fluid and the brain are shown in Figure 3 The penicillin concentration is seen to be higher in the brain than in the cerebrospinal *fluid in the controls The opposite is the case in animals with meningitis This difference increased with the degree of meningitis Thus, the in*fection appears primarily to have influenced the blood-cerebrospinal barrier An increase in the penicillin concentration in the cerebrospinal fluid was matched by an increase in the brain and *vice versa* If the animals are compared with one another, we find that the increase of the *concentration in the cerebrospinal fluid matched by an increase in the* brain was constant and independent of the degree of meningitis

The number of leukocytes in the cerebrospinal fluid of the rabbits with meningitis varied between 660 to 26,160 and the number of red corpuscles between 120 and 5,580 per millimeter The leukocyte count *in the control animals was generally lower than 100 per millimeter,* although it was 460 per millimeter in two cases The erythrocyte count in the controls was in general the same as in the experimental animals

If we examine the relation between the concentration of penicillin, on the one hand, and of leukocytes and erythrocytes in the cerebro*spinal fluid,* we find *good correlation between the concentration of penicillin and of leukocytes* in the cerebrospinal fluid of the rabbits with meningitis (Fig 4) This relation appears to be due to the presence of varying degrees of meningitis There is no such correlation in the control rabbits or within the various meningitis groups No correlation

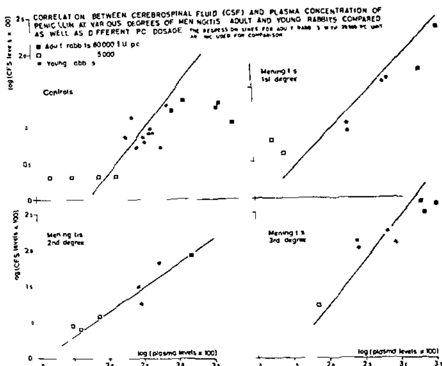



*Fig* 5

whatever could be found between the penicillin concentration and the concentration of erythrocytes

Figure 5 illustrates the correlation between the concentration of penicillin in cerebrospinal fluid and in plasma in adult rabbits which had received injections of 5,000 units of penicillin and in those which had received doses of 80,000 units per kilogram, as well as in young animals which had been given 20,000 units These correlations are compared with the regression line in the group of adult rabbits which had been given 20 000 units In this connection, consideration is given to the relationship between the values recorded for the three first-mentioned groups to the regression line The comparison was made for each one of the different clinical groups and for the controls The groups are small, but some general trends can be observed For example, the values obtained with a dosage of 5,000 units, reveal a consistent tendency to range above the regression lines, while the values obtained with a dosage of 80,000 units invariably are below these Thus the penetration of the barrier obtained with a dose of 80,000 units per kilogram was definitely inferior to the one obtained with 20 000 units and *penetration* obtained with a dose of 5,000 units per kilogram was superior to the one obtained with a dose of 20,000 units, bearing in mind the degree

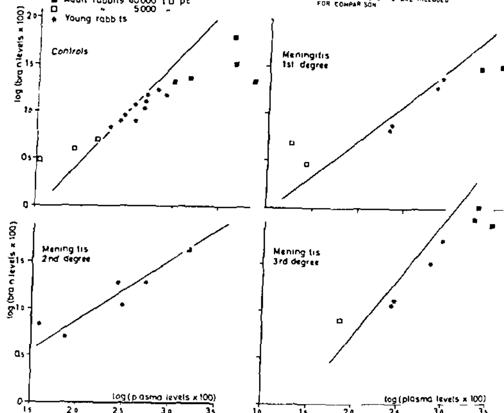



*Fig 6*

of meningitis The degree of resistance of the barrier increases with the penicillin doses – Seven cases of young animals were above the regression line, 14 were below, consequently there is no definite trend for young animals

A similar study was made on the relationship between the penicillin concentration in the brain and in plasma (Fig 6) The correlation was the same as between cerebrospinal fluid and plasma, with the exception that the penetration into the brain in young animals appeared to be consistently inferior to the one seen in adult animals (the levels were below the regression line)

Microscopic examination of specimens of the meninges removed from the rabbits confirmed the clinical findings For example, the extent of the anatomic changes in the cerebral meninges in the individual rabbits was usually proportional to the clinical degree of the meningitis Inflammatory changes were not found in the meninges of the controls Faint signs of irritation of the meninges were seen in a few of the controls

DISCUSSION

The question whether penicillin permeates cerebrospinal fluid is of interest to the therapy of infections in the cerebral nervous system. With intact meninges the blood cerebrospinal barrier is believed to prevent permeation of penicillin except to a minor degree of no therapeutic significance. With purulent inflammation of the meninges in man it has been found that penicillin permeates the cerebrospinal fluid with much greater facility. This is considered to be due to the fact that the blood cerebrospinal fluid barrier has been rendered at least partially ineffective by the meningitis as a result of the inflammation.

In the present investigation it was found that the concentration of penicillin in the cerebrospinal fluid rises when the concentration in plasma increases. This applied equally to rabbits with meningitis and to control rabbits.

That the penicillin concentration in cerebrospinal fluid tended to be higher in young than in adult rabbits both in the experimental animals and in the controls was probably due to the fact that the penicillin concentration in plasma was higher in young animals. Consequently there was not necessarily any difference between young and adult animals with regard to the passage of penicillin to cerebrospinal fluid. One objection to the investigation may be that the young animals should have been still younger. Experiments with younger animals were a failure however because of technical complications.

To discuss the correlation between the concentration of penicillin in cerebrospinal fluid and in plasma a comparison should preferably be made at a suitable time after the injection of penicillin. It appears from Part 2 of the present study that the passage of penicillin to cerebrospinal fluid is largely completed after about 15 minutes. An interval of 30 minutes between the administration of penicillin and the taking of samples appears to be appropriate for purposes of comparison.

The penicillin concentration in the cerebrospinal fluid was considerably higher in the animals with meningitis than in the controls. An explanation of this is that the inflammation in the meninges in the experimental rabbits facilitated the passage of penicillin into the cerebrospinal fluid. Evidence in support of this explanation is that with a certain concentration of penicillin in the plasma the concentration in the cerebrospinal fluid increased with the clinical signs of meningitis. That the passage was relatively better with smaller doses of penicillin and at the same time also with a lower penicillin concentration in the plasma than with larger doses may have been due to the fact that the passage into the cerebrospinal fluid was not completely uninhibited despite the presence of meningitis.

The fact that the penicillin concentration in the brain generally was higher in rabbits with meningitis than in healthy rabbits may indicate that in the former some penicillin had passed from the cerebrospinal

fluid to the brain substance No proof of this was obtained, however, by the multiple correlations (Table 3) A more probable explanation of the difference between the rabbits with meningitis and the healthy rabbits in this respect is that the blood-brain barrier also is altered in meningitis but not to be the same extent as of the blood-cerebrospinal fluid barrier The change in the former barrier was, however, great enough to result in a higher penicillin concentration in the brain substance than in rabbits without meningitis

The circumstance that the increase in penicillin concentration in the brain which occurs when the concentration in plasma rises was relatively independent of the degree of meningitis substantiates the theory that the penicillin passes into the brain largely directly from the blood and to a smaller degree *via* the cerebrospinal fluid This view is further supported by the fact that the penicillin concentration was higher in the brain than in the cerebrospinal fluid in the control animals and lower in the brain than in the cerebrospinal fluid in the animals with meningitis

The penicillin concentration in the cerebrospinal fluid appeared to have a certain correlation with the concentration of leukocytes in the cerebrospinal fluid This relationship must have been produced by the meningitis, since the penicillin itself was found not to influence the concentration of leukocytes In many of the control rabbits the concentration of leukocytes in the cerebrospinal fluid had increased, indicating that the measures taken had caused irritation of the meninges This irritation was not sufficiently pronounced, however, to facilitate the passage of penicillin into the cerebrospinal fluid Nor did it suffice to elicit even the slighest clinical signs of meningitis

The almost complete correspondence between the intensity of the clinical signs of meningitis and the degree of inflammatory changes revealed by microscopic examination of the cerebral meninges indicates that the passage of penicillin into the cerebrospinal fluid is related to the pathological change in the cerebral meninges

In the antibiotic treatment of infections of the meninges, it is very important that the preparations used reach a sufficiently high concentration in the cerebrospinal fluid In the present experiments a penicillin concentration in the cerebrospinal fluid which might be valuable in some of the ordinary forms of bacterial meningitis was obtained in rabbits with staphylococcic meningitis using intravenous doses of only 5,000 units per kilogram of body weight Using a dose of 20 000 units per kilogram, the penicillin concentration would be adequate for the majority of the infections with which we may be faced When these doses were used in control rabbits, the concentrations in the cerebrospinal fluid were too low to be of therapeutic significance Not until the dose was raised to 80,000 units per kilogram did the concentrations in the cerebrospinal fluid of control animals rise to levels of some therapeutic value

When an antibiotic has been selected for use in a given case, the dosage is determined with due regard to the sensitivity of the bacteria in question and to the site and severity of the infection It is not possible to follow the concentration of, for example, penicillin in the cerebrospinal fluid in cases of meningitis in man If the results obtained in rabbits correspond to conditions in man, a study of the penicillin concentration in the blood at an appropriate time might provide guidance for the penicillin treatment of staphylococcic meningitis and of other purulent forms of the condition In this connection it must be borne in mind that the penicillin concentration in cerebrospinal fluid does not increase proportionately with the concentration in the plasma, i e with the size of the dose, but that relatively less penicillin passes into the cerebrospinal fluid as the dosage is increased It is an advantage in clinically grave cases of meningitis that the passage of penicillin into the cerebrospinal fluid occurs more readily than in less serious cases of meningitis

In rabbits with meningitis only a dose of 20,000 units of penicillin per kilogram gave such high concentration in the brain as might give hopes of a therapeutic effect in infections susceptible to penicillin With a dose of 80 000 units per kilogram the penicillin concentration in the brain would be considerable in the experimental animals and relatively good in the control rabbits

Since bacterial infections in the brain substance usually occur in the form of abscesses, rather isolated from the rest of the brain, it is not certain that penicillin not even in high concentrations in the other parts of the brain will reach the centers of infection We cannot be sure that the walls of abscesses are permeable by penicillin, even though according to some workers, penicillin has a greater capacity of penetrating walls of infected lesions than most other antibiotis

It is therefore reasonable to assume that infections in the brain substance require higher dosages of penicillin than meningitis with the same bacterial strain and also that a brain abscess is relatively nonresponsive to treatment with penicillin This is in accordance with the view based on experience that brain abscesses are most successfully treated by surgery

## SUMMARY

(1) The passage of penicillin administered intravenously *into* the cerebrospinal fluid and the brain was studied in rabbits with staphylococcic meningitis and in healthy rabbits

(2) The penicillin concentration in the cerebrospinal fluid was higher in rabbits with meningitis than in animals without meningitis This also applied to the penicillin concentration in the brain The *penicillin* concentration in the cerebrospinal fluid and the brain in both of these categories varied with the variations of the penicillin concentration in the plasma

(3) The penicillin concentration in the cerebrospinal fluid following the same dose was relatively higher the more pronounced were the clinical signs of meningitis It also was related to the concentration of leukocytes in the cerebrospinal fluid

(4) The passage of penicillin to the brain appeared to occur mainly directly from the blood

(5) The penicillin concentration in the brain of rabbits with meningitis was somewhat higher than in the controls, in both of the categories it was relatively independent of the intensity of the symptoms of meningitis

(6) Penicillin passed into the cerebrospinal fluid with approximately the same facility in adult rabbits and in young animals

(7) If the results obtained were to serve as guidance in the treatment of meningitis in man, they might constitute an argument in favour of high dosage of penicillin with concomitant high penicillin concentrations in the plasma

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FROM THE MICROBIOLOGICAL DIVISION, THE ROYAL PHARMACEUTICAL INSTITUTE, STOCKHOLM, SWEDEN

# COMPARISON BETWEEN AN IN VITRO INDUCED PENICILLIN-G-RESISTANT STRAIN OF STAPHYLOCOCCUS AUREUS AND THE CORRESPONDING PARENT STRAIN

## I. *Immunological Studies*

*By*

BIRGITTA NORKRANS and LENNART WAHLSTRÖM

Received 21.vi.62

For several reasons, increasing interest has been focused on the antigenic properties of Staphylococcus aureus. In his extensive monograph on Staph. pyogenes, *Elek* (1959) devoted some chapters to the problem, and *Oeding* (1960) has recently presented a review of the subject.

The antigenic properties of Staph. aureus are considered to be complex, since cross reactions are extremely common, which may be due to the sharing of antigens in varying proportions among different strains. *Cowan* (1939) used heat-killed bacteria for immunization, and was able to distinguish 3 main types by absorption and slide agglutination. *Christie & Keogh* (1940) increased the number of types to 9, and *Hobbs* (1948) to 13. *Oeding* (1952), using factor sera produced against formalin killed bacteria, was able to demonstrate 10 different antigens in pyogenic staphylococci (*Haukenes et al.* 1960, 1961). It is usual to speak of major and minor antigens rather than of type specific antigens. Although much work has been done in this field, no generally accepted serological typing system has been forthcoming.

Knowledge of changes in the antigenic patterns due to acquirement of penicillin resistance should be of some interest, but little research has been done on the subject. *Oeding* (1952) found evidence of a correlation between sensitivity to penicillin and serological type. Cowan's type III with the phage patterns 6/7/47 is supposed to be an easily mutating group. Recently, however, staphylococci of all phage patterns have been found to yield penicillin-resistant mutants (*Barber & Burston* 1955).

*Murray et al.* (1959), in electron-microscopical studies, compared the appearance of the cell wall of a penicillin-sensitive and corresponding penicillin resistant staphylococcal strain and showed the latter to be

more thin. A comparison between sensitive strains (Cowan's types I, II, and III) and corresponding penicillin-resistant ones has been performed by *Stern & Elek* (1957), using agglutination reactions with absorbed antisera produced against heat-killed bacteria. They were not, however, able to find any changes in the antigenic structure of penicillin-resistant variants.

An account is given in the present paper of a comparative study, by means of the agar diffusion technique, between a clinical penicillin-G-sensitive strain and a corresponding penicillin-resistant one, prepared in vitro, with respect to possible antigenic changes. No attempt has been made to analyze the precipitation lines chemically but we have confined ourselves to recording their number.

## MATERIALS AND METHODS

### *Organism*

A coagulase-positive strain of Staphylococcus aureus was chosen for this study. This strain, with the clinical number 1429, was isolated from pus from middle ear, and has also been used in a preliminary study (*Norkrans & Bertrandsson* 1962).

### *Preparation of Penicillin-G-resistant Strain*

The penicillin used was the sodium salt of benzylpenicillin, kindly placed at our disposal by Kabi Co. The resistant strain was prepared from an one-cell culture of 1429, initially by the gradient plate technique of *Szybalski* (1952). However, when cultivation was to be performed in a liquid medium, the resistant strain was also prepared in it, by repeated transfers to penicillin in rising concentration. Serial dilutions of penicillin were used, dissolved in sterile phosphate buffer, pH 6.0. The medium consisted of Antibiotic medium 3 (Bacto-Penassay Broth Difco). A continous check was made for contaminations.

The resistance capacity obtained in this strain (R) was 6000 $\mu$g of penicillin/ml. The strain was lyophilized, and stored in sealed ampoules.

### *Cultivation Technique*

The organisms were grown for 20 hours at 37° C in 12 litres of the aforementioned liquid medium in a 15 litre-flask, through which air was continously bubbled. To increase the yield, 1 per cent glucose was added and a small quantity of Antifoaming DCA to prevent foaming. The flasks were inoculated with three 300-ml Erlenmeyer flasks, each containing 50 ml of a 20-hour culture. Before inoculation, the resistant strain, from the ampoules, was transferred several times in the highest possible penicillin concentration with a view to preventing the consequences of back mutation (*Eriksen* 1946), since no penicillin was added to the cultivation flasks. The bacterial yield was about 5.7 g dry weight of bacteria in the sensitive strain (S) and about 2.5 g in the resistant one (R). It was collected in a continous-action rotor attached to a high-speed 17 centrifuge (MSE, Ltd.), washed three times with sterile saline, lyophilized, and stored in a refrigerator.

### *Antigen for Immunization*

Antigen for immunization was prepared in two different ways, i.e. by heat treatment and by formalin treatment. The latter was suggested by *Oeding* (1952) as [illegible] *the antigenic structure of pyogenic staphylococci* [illegible] f lyophilized bacteria (0.5 g/10 ml) was treated at [illegible] or 6 hours, since the bacteria were not completely [illegible] treatment. The cells were washed three times with sterile saline, lyophilized, and [illegible] eighed.

Formalin treatment. Two per cent formalin in saline was used to kill the cells, during mechanical shaking for 1 hour at 37° C. The subsequent procedure was as above.

Two bacterial suspensions in saline were prepared they contained 1 and 5 mg dry weight per ml respectively The vaccines were tested for sterility

*Antisera*

Male rabbits weighing from 2 2 5 kg were used for the production of antisera Three rabbits were used for each strain They were controlled for the absence of antibodies to the experimental strain by pre immune sera It can be mentioned that the latter were always free from "normal antibody (cf *Oeding* 1960) The vaccine was injected intravenously four times a week for one month in a successively increasing dose each week i e 1 2 5 and 10 mg One week after the last injection the rabbits were exsanguinated by cardiac puncture The following antisera were prepared from whole blood with *Cohn s* (1952) technique $S_H$ = antiserum against heat killed sensitive Staph aureus (S) $R_H$ = antiserum against heat killed resistant staphylococci (R) $S_F$ = antiserum against formalin killed S $R_F$ = antiserum against formalin killed R

*Antigens for Diffusion Tests*

A Disintegration in glass homogenizer 0 200 g of lyophilized bacteria were suspended in 10 ml of sterile saline The suspensions were treated manually for 10 minutes in a glass homogenizer Intact cells and fragments of cell walls were seperated by centrifuging for 20 minutes at 13 000 g The antigen solution obtained was preserved with merthiolate (1 10 000)

assembly being kept cold in an icebath throughout exposure Remaining intact cells and bacterial debris were seperated by centrifugation for 20 minutes at 13 000 g Merthiolate (1 10 000) was added as preservative to the antigen solution obtained The solutions were tested for protein content with the same method as in A and for the same reason

*Ring Test*

The tests were made ... of the antigen ... Readings were ... proved useful ... ments

*Agar diffusion tests*

We used the methods devised by *Ouchterlony* (1949 a b c 1953) The gel con

was 0.3 ml and the Petri dishes used had diameters of 5 cm After filling they were placed at 37° C in a moist atmosphere in order to avoid refilling (cf *Kaminski* 1954) Readings were made daily f r 10 days

## RESULTS AND DISCUSSION

### A *Antigen Antibody Reactions with Antigens Prepared in a Glass Homogenizer*

*Oeding* (1953) suggested that blocking and heat labile antigens occur on the bacterial surface, whereas heat stable and blocked antigens are

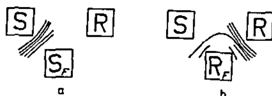



*Fig 1*
Precipitation patterns of strain 1429: antigen solution prepared by ultrasonic disintegration
S = sensitive strain $S_F$ = antiserum against formalin-killed S
R = resistant strain $R_F$ = antiserum against formalin killed R

present in the protoplast An antigen preparation obtained by grinding in a glass homogenizer presumably consists mainly of protoplast antigens and may be devoid of antigens derived from the robust staphylococcal cell wall The antisera to formalin-killed staphylococci ($S_F$ and $R_F$) contain antibodies to the antigens on the bacterial surface, in addition to both heat-stable and heat-labile protoplast antigens

Surprisingly enough, antisera against heat-killed staphylococci, $S_H$ and $R_H$, gave one precipitationline more on the agar plates than $S_F$ and $R_F$ A possible explanation is a relative increase in the heat-stable antigens during heat treatment, since – in preparation of antigen for immunization – the loss in weight of the heat-killed bacteria exceeded that of the formalin-killed ones by about 20 per cent Moreover, formalin treatment is supposed to bind the superficially located antigens very firmly to the bacterial body (cf *Jensen* 1959) Since, however, as a whole very few precipitation lines were obtained and the tests did not reveal any shared antigens for the sensitive and the resistant strain, it could be supposed, that the homogenizing effect of grinding in a glass homogenizer was too weak to produce a sufficient concentration of the antigenic material actually present in the protoplast Consequently, the ultrasonic disintegration method was applied

B *Antigen-Antibody Reactions with Antigens Prepared by Ultrasonic Disintegration*

A larger number of diffusible antigens was obtained by preparation with ultrasonic waves When they were tested with $S_H$ and $R_H$ (Fig 2) and with $S_F$ and $R_F$ (Fig 1), respectively, more precipitation lines were obtained in the latter case

The reaction with $S_F$ (Fig 1, a) indicated that the sensitive strain and the corresponding induced penicillin-resistant one had no antigens in common Nor did the resistant strain and $S_F$ at any concentration give any precipitate in the ring test

In tests with $R_H$, on the contrary, the resistant and the sensitive strains seemed to share at least two antigens (Fig 1, b) The fact that the sensitive strain did react with antiserum against the resistant one may indicate that these shared antigens were actually present in the

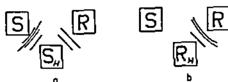



*Fig 2*

Precipitation patterns of strain 1429 antigen solution prepared by ultrasonic disintegration

S = sensitive strain $S_H$ = antiserum against heat killed S
R = resistant strain $R_H$ = antiserum against heat killed R

former strain, but that the concentration was too low to give rise to any detectable amount of antibodies This implies that the sensitive and the resistant strain had two antigens in common, although their concentration differed in the two strains

As seen in Fig 2, the opposite results were obtained in diffusion tests with antisera to heat killed bacteria Thus, no lines occurred between the sensitive strain and $R_H$ (Fig 2, b), whereas weak precipitation lines were present between the resistant strain and $S_H$ (Fig 2, a) An explanation analogous to the one suggested above, however, might apply in this case as well, *i e*, that the heat-stable antigens were present in a too low concentration in the resistant strain This strain, in fact, was found to have fewer precipitation lines (cf Figs 1 and 2), indicating that it is more sensitive to heat treatment than is the parent strain

On the assumption that the ultrasonic method gives protoplast antigen alone (*Cohen et al* 1958), the increase in number of precipitation lines should be an expression only of the greater efficiency to be expected with this method It cannot be ruled out, however, that they might have derived from some splitting off and degradation of the protoplast antigens (cf *Cohen et al* ) Although the ultrasonic method may be a gentle one, it may involve a certain risk for the antigenic material A prolongation of treatment, for instance, brought about a disappearance of some lines in the agar-diffusion test Thus, exposure for 15 minutes the time used throughout this investigation – resulted in six precipitation lines (strain 1429 R), whereas, only 5 and 4 lines were obtained after exposure for 30 or 60 minutes, respectively

*Salton* (1960) emphasized that "the disintegration by sound and supersound can lead to a greater breakdown of the wall structure than that encountered with other disintegration methods commonly used Even the robust walls of Staphylococcus aureus can be rendered non-sedimentable" This statement argues in favour of another probable explanation of the larger number of precipitation lines in our tests of antigenic material prepared by ultrasonics, as compared to that prepared by the glass homogenizer method *i e*, that some of the additional lines derived from cell-wall material

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tant one (Figs 1 and 2), shows that qualitative as well as quantitative differences were present Thus, *more of the antigens obtained from the resistant strain than from the sensitive one were heat-labile* Furthermore, the detectable heat-labile antigens occurred in higher concentration in the resistant strain than in the sensitive *one*, whereas *the opposite* applied to the heat-stable antigens Our results therefore differ from those obtained by *Stern & Elek* (1957), who stated that "penicillin- and streptomycin-resistant variants show no changes in the antigenic structure"

Different explanations of this discrepancy can be discussed Our resistant strain 1429 had a very high resistance capacity (6000 $\mu$g ml) whereas those used by the aforementioned authors had a very low one (2 IU/ml) This difference, however, does not seem to be crucial per se, since four penicillin-G-resistant staphylococci, with resistance capacities ranging from two to 80 $\mu$g/ml, all deviated from their sensitive parent strains as regards the antigenic pattern (*Norkrans & Bertrandsson* 1962)

As a rule penicillin-resistant strains, induced in vitro, are not penicillinase-producers (cf *Wallmark* 1953) It is valid for 1429 too In this respect the strains, used by *Stern & Elek* might differ from ours However, they do not mention, that their strains were induced under any special conditions, which – according to *Szybalski* (1953) – should favour the appearance of penicillinase-producers

The discrepancy, however, may be explained as follows *Most of the* differences found here, and relating to the heat-labile antigens, were detectable in view of the fact that we used antisera against both heat-*killed and formalin-killed bacteria*, whereas *Stern & Elek used* heat-killed ones only Furthermore, they used the agglutination method, and not the agar-diffusion technique used in the present study The former *may disclose pronounced differences between* superficially located antigens, whereas the latter allows even exceedingly slight differences in chemical costitution to be detected and, in addition, not only those limited to the surface antigens


SUMMARY

(1) The antigenic pattern of a clinical, penicillin-sensitive, *coagulase*-positive strain of Staphylococcus aureus (no 1429) has been compared with that of the corresponding penicillin-resistant strain, induced in vitro

(2) The reactions between antigens prepared in a glass homogenizer, as well as by exposure to ultrasonic waves and antibodies produced against heat-killed and formalin-killed bacteria have been tested by the double-diffusion agar technique

(3) Both qualitative and quantitative differences were observed between the antigenic pattern of the resistant strain and the pattern of

the parent strain It is suggested that these changes must have arisen on acquisition of penicillin resistance

(4) The ultrasonic method, its efficiency and its probable effect on protoplast and cell wall are discussed, as well as the probable degradation of the antigenic structure produced by it

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# A SIMPLE BIOCHEMICAL "TRIPPLE TEST" FOR PRELIMINARY IDENTIFICATION OF GROUP A STREPTOCOCCI

*By*

ALF WALLERSTRÖM

Received 26.xi.62

In the grouping of streptococci the practical purpose of which is to differentiate pathogenic strains from others the serological methods of *Lancefield* and *Griffith* have now largely superseded the previous methods based on fermentation reactions etc. Several tests are however available which are suitable for routine diagnostic work and will bring about a preliminary classification in a simpler way than by the use of serological methods.

As known most of the $\beta$ haemolytic streptococcal strains which are pathogenic to human beings belong to one of the Lancefield groups A, C or G and to group A in particular. It is also known that addition of nucleinate to the culture medium almost invariably stimulates the production of haemolysin by strains belonging to these groups; this response may be used as criterion for primary classification of the strain as pathogenic to human beings (*Grubb* 1955). Another simple test now widely used is based on the fact that most streptococci of group A are susceptible to bacitracin while streptococci of other groups are usually resistant (*Maxted* 1953).

Addition of glucose to the culture medium lowers the haemolytic activity of certain $\beta$ haemolytic streptococci, particularly of group A streptocci. This phenomenon has received little space in the anglo-american literature and it was the fortuitous observation of such depression of haemolysis in our laboratory which prompted the present investigation.

The phenomenon has been known for a long time and was described as early as in 1906 by *Ruediger*. In 1940 *Kobayashi* offered a classification system for $\beta$ haemolytic streptococci; the system included a group I which was most commonly isolated from cases of scarlatina and from pyogenic lesions and whose haemolytic activity was depressed by glucose. The author himself stressed the similarity between his group I and *Lancefield's* group A, and in a later paper *Ogawa & Kobay-*

*ashi* (1941) reported that strains of group I regularly belonged to group A *Hackenthal & Bierkowski* (1951) published another classification system based on a series of blood agar plates to which had been added various sorts of sugar, *i a* glucose According to *Bierkowski* (1956), the absence of haemolysis by strains cultured on glucose blood agar is absolute evidence of group A streptococci, streptococci of groups B, D and N also lose some of their haemolytic activity but they also produce a characteristic discoloration of the blood agar – *Schneweis & Nikfetrat* (1959) compared the results of serological determination of a number of streptococcal strains with those obtained with bacitracin and glucose, all of their 81 group A strains were susceptible to bacitracin and showed inhibition of haemolysis on glucose medium, while strains belonging to group B, C and G showed no such uniform pattern

Neither the German nor the Japanese authors could find any relation between the reaction of the streptococci to glucose in blood agar medium and their capacity to ferment different carbohydrates, including glucose As early as 1919 *Brown* expressed the view that absence of haemolysis by streptococci on addition of glucose to the medium was due to reduced formation of haemolysin In several of his papers on the preparation of streptolysin, *Todd* states that if streptococci are allowed to grow in medium containing glucose (*e g Todd-Hewitt* broth), no streptolysin S will develop but only streptolysin O (*Herbert &Todd* 1944), *this has since been confirmed by other workers* (*Bernheimer & Rodbart* 1948)

In the present investigation, streptococcal strains belonging to differt serological groups were studied with a "tripple test" composed of three sub-tests testing for susceptibility to bacitracin, stimulation of haemolysis by nucleate, and inhibition of haemolysis by glucose, the purpose was to find out if group A streptococci could be differentiated from other streptococci in this way

## MATERIAL AND METHODS

*Material* 142 β haemolytic streptococcal strains of which 125 had been collected from specimens sent to the laboratory (throat and wound infections gynaecological samples and urine samples) and the remaining consisted of reference strains 18 strains which had given growth of characteristic colonies on tellurite agar were classified as enterococci 85 strains were determined serologically at the Strepto [illegible] ology Malmo by the method described by [illegible] the method of *Thulin* (1949) The number [illegible] en in Table 1

*Medium* The streptoc[illegible] d on blood agar plates the blood content of which consisted of equal amounts of horse and sheep blod and which contained small amounts of *KCl* $Na_2HPO_4$ *and maltose* (*Grubb & Nyman 1955*) For the performance of the test was also used

*for the bacitracin test* discs (diameter 5 mm) containing 0 2 F bacitracin prepared by the Bacteriological Laboratory Karolinska Sjukhuset Stockholm

*for the glucose test* discs (diameter 12 7 mm) manufactured by *Schleicher & Schüll* and a 10 per cent glucose solution (Dextropur) which had been heated in a water bath at 100° C for 20 minutes

*for the nucleate test* tablets of the following composition natr nuclein e faece (Merck) 0 025 talcum 0 0236 magn stearas 0 0004, amyl solan 0 065

*Performance* Each strain was smeared densely on a blood agar plate, after which the two discs and the nucleate tablet were placed on the surface of the agar The disc used for the glucose test was then allowed to absorb 3 drops of glucose solution after which the plate was incubated at 37° C The results were read at the earliest 12-14 hours later

Some strains were also studied by two other methods for their reaction to glucose 87 strains were smeared on the blood agar plates of the above mentioned composition *but with an addition of 3 per cent glucose to the medium 76 strains* were tested with a glucose solution in stamped holes in the plate (agar-cup method) All three methods gave identical results, upon glucose agar medium many strains did not loose all their haemolytic power but the resulting haemolysis was faint and greenish somewhat resembling the $\alpha$ haemolysis given by viridans streptococci

*Evaluation*

*Bacitracin test* positive (strain susceptible), if a zone of growth inhibition at least 12 mm in diameter occurred around the discs

*Glucose test* positive, if a zone free of haemolysis without discoloration of the medium occurred around the discs.

*Nucleate test* positive if the haemolysis was definitely stimulated around the tablet (=the individual streptococcal colonies showing wider and more brilliant *zones of haemolysis)*

*Tripple test* positive only if all three sub tests were positive

## RESULTS

In Table 1 is shown how the 142 streptococcal strains belonging to 13 different serological groups reacted to the "tripple test" (examination of the susceptibility to bacitracin and the effect of nucleate and glucose upon the haemolysis)

TABLE 1

*Susceptibility to Bacitracin and Influence of Glucose and Nucleate upon the Haemolysis of Streptococci of Different Serological Groups*

| Group | nc+ gl+ bac+ | nc+ gl+ bac | nc+ gl bac+ | nc+ gl− bac− | nc gl+ bac+ | nc− gl+ bac− | nc− gl− bac+ | nc− gl− bac− | n |
|---|---|---|---|---|---|---|---|---|---|
| A | 53 | | | − | − | − | | − | 53 |
| B | | − | | | | − | | 2 | 2 |
| C | | 2 | | 2 | | 3 | | − | 7 |
| D | | | | − | | − | | 19 | 19 |
| E | | − | | 1 | | − | − | 2 | 3 |
| G | 1 | 5 | 1 | 4 | − | − | 1 | − | 12 |
| H | − | 1 | − | 2 | | − | − | 1 | 4 |
| K | | − | | − | − | − | − | 1 | 1 |
| L | | 1 | − | − | | − | 1 | − | 2 |
| M | | 1 | − | 1 | − | − | 1 | 4 | 7 |
| N | | | | | | − | | 1 | 1 |
| O | | | | − | − | − | − | 3 | 3 |
| P | | | − | − | 1 | − | − | 1 | 2 |
| non A | | 2 | − | 6 | − | 5 | | 13 | 26 |

nc+ = haemolysis stimulated by nucleate
gl+ = haemolysis inhibited by glucose
bac+ = susceptible to bacitracin

The 53 group A strains studied proved to be susceptible to bacitracin, their haemolysis was inhibited by glucose and stimulated by nucleate the "tripple test" thus positive One strain not falling within group A, a group G streptococcus, showed the same pattern

The material included 19 strains of groups C and G Of these, 8 (42 per cent) gave a positive reaction to glucose *as well as* to the nucleate tests, while 18 (95 per cent) gave a positive reaction to one of these two tests As to streptococci not falling within the A-C-G groups (44 strains), the corresponding figures were 3 (7 per cent) and 8 (18 per cent), respectively

6 strains not belonging to group A (6 7 per cent) gave a positive bacitracin test No streptococcus of groups B, D, K, N and O gave positive reaction to any of the tests

The reading of the "tripple test" usually caused no difficulty Some streptococcus strains, particularly those belonging to groups C, G, and L, primarily showed very wide and brilliant zones of haemolysis, which made it difficult to recognize any stimulation of haemolysis – Some strains produced discoloration of the blood agar medium around the glucose discs Pronounced and constant discoloration of the substance of the medium was thus produced by most strains of groups B, M, N and O, a weaker and less regular discoloration by enterococci and by some strains of groups C, G, H, and L A darkening of the medium can, of course, at first glance be confused with inhibition of haemolysis, the discoloration zones were, however, always diffusely outlined, in contrast to the circular and well defined zones of inhibition of haemolysis, so that, as a rule, evaluation was not difficult

## DISCUSSION

The nucleate- and glucose tests influence the same function of the streptococci, the formation of haemolysin, and it is probably the formation of streptolysin S that is affected It is known that the nucleate stimulates the haemolytic activity of groups A, C, G, E and L (*Grubb* 1955), and in this material it was also found to stimulate this activity of some strains of group H and M *Bernheimer* (1948, 1949) found the nucleic acid haemolysin in all respects studied identical with streptolysin S, he observed the nucleic acid effect in strains of groups A, D, E, G, H and L

The glucose reaction has been tested by all previous investigators by addition of glucose (1–3 per cent) to the culture medium The disc method described appears to be simpler and to give more easily readable results, in work with glucose blood agar it is necessary, for example, to use only very small inocula, since any preformed haemolysin will otherwise be transferred with the platinum loop and produce haemolysis also in the glucose medium (performed haemolysin is thus not inhibited by glucose) – In the present study the glucose was poured

onto the discs just before use, but glucose discs may be prepared in advance, and they will stand several months' storage They can probably be stored much longer, but they are more liable to infection than antibiotic discs

Neither the glucose nor the nucleate test alone can decide whether or not a streptococcus belongs to group A The bacitracin test is more reliable, in previous investigations the frequency of group A streptococci among resistant strains has been about 1 per cent, while the frequency of non—A—streptococci among sensitive strains in 8 different series was on the average 3 6 per cent (*Schneweis-Nikfetrat*) The corresponding figures in the present material were O and 6 7 per cent, respectively It thus seems to be more reliable that a streptococcus which is resistant to bacitracin is not a group A strain than vice versa

The "tripple test" appears to be a more reliable method than testing with bacitracin alone, in the present material only 1 "false" group A streptococcus was noted, and no "false" non-A-streptococci If, primarily the question is not whether a streptococcus belongs to group A, but rather whether it is pathogenic to human beings, the test has a further advantage a positive reaction on glucose and nucleate argues for the strain belonging to one of the human pathogenic groups C or G, which are not at all recognizable with the bacitracin test

## SUMMARY

Non-serological methods can be used for primary distinction between group A and other $\beta$ haemolytic streptococci Susceptibility to bacitracin and stimulation of haemolysis by nucleate are characteristic properties of group A streptococci, as is another less known phenomenon inhibition of haemolysis on addition of glucose to the medium, which is probably due to lowered production of streptolysin S Each of these phenomena occur also in streptococci not falling within group A, but non A streptococci rarely show both susceptibility to bacitracin, stimulation of the haemolysis by nucleate, and inhibition of haemolysis by glucose All of the 53 group A streptococcal strains studied had these three properties, while 88 of 89 non-A-streptococcal strains (representing all serological groups except F and Q) possessed at most two of them 95 per cent of the streptococci belonging to groups C and G reacted positively to glucose or nucleate, this implies an advantage of the "tripple test" particularly over testing with bacitracin alone, which does not show whether the streptococci belong to group C or G Streptococci of groups B, D, N and O gave negative reactions in all of the three subtests

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# THE AETIOLOGY OF RESPIRATORY TRACT INFECTIONS IN MILITARY PERSONNEL

## 4 The Recovery of Coxsackie A-21 Virus from Cases with Minor Respiratory Disease

By

REBEKKA KJERSGAARD, G LINDBOM, Z DINTER and L PHILIPSON

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Some enteroviruses have been associated with respiratory disease in children (1,2) and military recruits (3, 4, 5, 6), although few controlled studies linking the virus to the illness have been reported

The Coe virus, originally isolated by *Lennette et al* (4) and later identified as Coxsackie A 21 (7), has been isolated in single instances in North America (4), England (8), Holland (9) and Japan (10) from recruits with mild respiratory disease Recently a controlled study was presented, linking the mild respiratory disease observed to the Coxsackie A 21 infection (5 6)

During the course of a continuous study of respiratory disease in military recruits a number of strains of Coxsackie A-21 virus were isolated during the autumn of 1961 The present investigation reports the clinical and virological findings in the personnel affected by this virus

### MATERIALS AND METHODS

The investigation was carried out in a military camp at Uppsala according to plans previously described in detail (11 13) Only conscripts reporting for respiratory disease at the hospital of the camp were examined and a controlled study has not yet been performed More detailed information about the techniques used has been reported earlier (11 13)

#### *Collection of Specimens*

[illegible] at [illegible] 10 day intervals The sera were stored at 20° C until tested

#### *Virus Isolation*

All virological specimens were inoculated into the various cell cultures used cynomolgus monkey kidney human kidney and HeLa cells. Source and maintainance of the cultures have been described (11) Cultures were placed in a rotating

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TABLF 1

*Virus Isolations in Military Recruits with Respiratory Disease*

| Total number of cases | Coxsackie A 21 Number of cases | | Herpes simplex Number of cases | Adenovirus type 2 Number of cases |
|---|---|---|---|---|
| | Throat | Faeces | | |
| 22 | 6 | 0 | 1 | 1 |

*Haemagglutination by Coxsackie A 21 Virus*

Five of the eight strains of Coxsackie A-21 virus isolated from the throat swabs in human kidney cultures gave a positive haemagglutination with human group 0 erythrocytes at 4° C It was found, however, that the haemagglutinating property of the strains was not stable, since some batches of human cells produced haemagglutinin when inoculated with the virus, but others did not support this property although virus was produced in titers of $10^{-6}$ $TCD_{50}$/ml This capacity was not infuenced by different media of the cell cultures

TABLE 2

*Demonstration of Antibodies against Coxsackie A 21 Virus and Other Viruses Recovered in Diseased Recruits*

| Case number | Virus isolated | Fourfold increase in neutralizing antibodies against Coxsackie A 21 | Fourfold increase in HI* antibodies against Coxsackie A 21 | Fourfold increase in CF antibodies against Herpes simplex | Fourfold increase in CF antibodies against adenovirus |
|---|---|---|---|---|---|
| 1 | Coxs A 21 | + | + | — | — |
| 2 | | + | + | — | — |
| 3 | Coxs A 21 | + | + | — | — |
| 6 | Coxs A 21 | + | + | — | — |
| 7 | Coxs A 21 | + | + | — | — |
| 8 | Coxs A 21 | + | + | — | — |
| 9 | Coxs A 21 | + | + | — | — |
| 19 | Herp simp | + | + | + | — |
| 22 | Adeno type 2 | — | — | — | + |

HI — Haemagglutination inhibition

*Serological Findings*

Sera from all cases were investigated for neutralizing and haemagglutination-inhibition antibodies against a number of viral antigens as described in Methods Table 2 shows the results obtained in the neutralization and HI tests for Coxsackie A-21 antibodies as well as the serological results in the cases from which Herpes simplex virus and ade

drum at 37° C and read for microscopic changes every second day for 10 days Negative cultures were blindly passaged and incubated for another 10 days Haemadsorption tests were carried out every fifth day on monkey kidney cultures

### *Virus Identification*

Cytopathic agents were identified by means of neutralization with specific antisera against the different viruses Identification was also confirmed by haemagglutination inhibition test (HI) with strains having haemagglutinating properties Sera used for identification of Coxsackie A 21 virus were obtained by immunization of guinea pigs The prototype strain of Coxsackie A 21 virus was kindly supplied by Dr *A Stedmyr* and an immune serum against this virus was supplied by Dr *D A J Tyrrell*

### *Bacteriological Technique*

The specimens were investigated according to techniques previously described (12) and potential pathogens of the respiratory tract as *Staphylococcus aureus* haemolytic streptococci *Haemophilus influenzae* and pneumococci were recorded

### *Haemagglutination (HA) and Haemagglutination inhibition (HI)*

HA and HI were carried out according to techniques earlier described for haemagglutination of ECHO viruses (14) Standardized 0 5 per cent blood suspensions of human group O erythrocytes were used and the test carried out at +4° C. Four HA units were used in the HI tests

### *Serology*

Neutralizing antibodies were determined by incubating serial twofold dilutions of serum with 100 $TCD_{50}$ of virus for 1 hour at 37° C and subsequently the mixtures were tested for cytopathic effects on human kidney cultures Complete suppression of the cytopathic effect was used in determining endpoints The HI test has been described previously (14) The titer in the HI test was expressed as the last initial dilution of serum which produced complete inhibition of haemagglutination Complement fixation tests were carried out in tubes with 0 1 ml of antigen and serum respectively according to a method previously described (11) All sera from the affected conscripts were examined for complement fixing (CF) antibodies against adenoviruses influenza A B and C viruses mumps and parainfluenza 1 and 3 viruses Herpes simplex and Ornithosis HI tests were also performed against parainfluenza 1 2 and 3 viruses The preparation of antigens has been described in a previous report (11)

Antistaphylolysin antistreptolysin and antipneumolysin were determined according to methods described previously (12) Complement fixing antibodies against *H influenzae* was determined with a sonicated extract of whole bacteria as the antigen

## RESULTS

### *Virus Isolation*

During the period September 1961 January 1962 only 22 recruits of a total of 710 conscripts reported for respiratory disease at the hospital of the military camp These cases occurred during the period October 23 November 29 In six of the cases Coxsackie A-21 virus was isolated in the two first cases observed, the virus was isolated both at onset and 5 days later In all of the positive cases the virus was only recovered from the throat and not from faeces In one case Herpes simplex virus and in another case adenovirus type 2 were isolated from the throat Table 1 shows the virus isolations from the 22 cases investigated

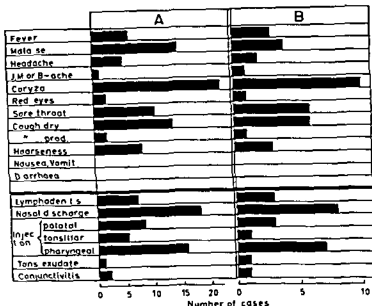



*Fig 1*

Symptoms and signs in recruits with respiratory disease A Total number of cases B Cases associated with Coxsackie A 21 infection J M or B ache = Joint Muscle or Boneache

the diseases observed were classified as minor respiratory disease as seen in Table 4 The classification used is based on the differentiation earlier described (13) and originally reported by *Grieble et al* (15)

TABLE 4

*Diagnosis of Respiratory Disease Observed during the Autumn of 1961*

| Diagnosis | Total number of cases | Number of cases associated with Coxsackie A 21 infection |
|---|---|---|
| Minor respiratory illness | 15 | 7 |
| Pharyngo-conjunctivitis | 2 | 1 |
| Exudative pharyngitis | 1 | 0 |
| Acute respiratory disease (ARD) | 4 | 1 |

## COMMENT AND SUMMARY

An association between Coxsackie A-21 virus and minor respiratory disease in military recruits appears likely from the presented data, but a definite linkage between the virus and the disease can only be obtained after a controlled study However, the combined results from the serological investigation with a number of viral and bacterial antigens indicate an association between the disease and the virus recovered

*novirus type 2 were isolated* It can be seen that in all cases from which the Coxsackie A-21 virus was isolated, a fourfold or higher increase in titers of neutralizing as well as haemagglutination-inhibiting antibodies was observed In additional 3 cases a significant antibody rise was demonstrated, although Coxsackie virus was not isolated from the cases Except for the reported positive findings, *no significant titer increases* were *observed* in the other tests performed

### *Bacteriological Findings*

The results of the bacteriological examination as well as *the significant* titer rises against the bacterial antigens are shown in Table 3 *Except for the 2 cases associated with infection of haemolytic* streptococci, bacterial infections did not appear to contribute to the clinical disease observed

TABLE 3
*Bacteriological Findings and Antibody Response to Bacterial Antigens in the Recruits with Respiratory Disease*

| Bacteria isolated at admission | Total number of cases (22) | Number of cases (9) associated with Coxsackie A 21 infection |
|---|---|---|
| Staph aureus | 11 | 4 |
| Haemolytic streptococci | 3 | 3 |
| Pneumococci | 1 | 1 |
| *H influenzae* | 2 | 1 |
| Meningococci | 1 | 1 |
| *Significant increase in antibody titer* | | |
| Antistreptolysin | 2 | 2 |
| Antistaphylolysin | 1 | 0 |
| Antipneumolysin | 0 | 0 |
| CF antibodies against *H influenzae* | 0 | 0 |

### *Clinical Findings*

The conscripts reporting for respiratory disease were examined by a physician in the team and symptoms and signs recorded The main symptoms were coryza, cough, sore throat, and malaise the signs observed were nasal discharge, injection of the pharynx, and in some cases fever above 38° C Fig 1 shows the symptoms and signs in the 22 cases observed in the autumn of 1961, compared with the 9 cases associated *with Coxsackie A-21* virus on the basis of virus isolations and/or significant increase in antibody titres against this virus When the cases were *distributed according to category of respiratory disease most of*

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# IMMUNO ELECTROPHORETIC STUDIES OF SERUM PROTEINS WITH PARTICULAR REFERENCE TO THE GC-SYSTEM IN EXCHANGE-TRANSFUSED NEWBORNS

*A Preliminary Report*

*By*

JAN HIRSCHFELD and BO A NILSSON

Received 15 ix 62

Recent studies on human serum proteins by means of starch-gel electrophoresis (12–14) and immunoelectrophoresis (1–9) have revealed the existence of proteins with varying electrophoretic mobilities in sera from different individuals Up to now, three independent protein systems, the haptoglobins (Hp), the transferrins (Tf) and the group-specific components (Gc) have been found to occur in electrophoretically distinct and genetically determined types in individual sera (2, 12, 13)

These serum protein differences can be visualized by means of immunoelectrophoresis since precipitates with varying electrophoretic positions and shapes appear, when sera belonging to different Hp-, Tf- or Gc-types are tested This fact allows sera to be divided into several main groups (2 9)

Sera which have a fast migrating group-specific component (Gc 1–1) give an arc shaped precipitate which is situated more anodal than that of sera with the slow component (Gc 2–2) In addition, certain human sera belong to a third main group (Gc 2–1) which, in immunoelectrophoresis gives a two peaked or extended precipitate (2) This shape of the Gc 2 1 precipitate can be explained by the simultaneous presence of the "fast" as well as the "slow" Gc protein in sera of this type Therefore a similar precipitate can be obtained by subjecting a mixture of Gc 1 1 and Gc 2 2 sera to immunoelectrophoresis (2, 6, 7) The anodal part of this extended precipitate is thus formed by the reaction of the electrophoretically fast Gc molecule populations with the Gc antibodies in the immune serum the cathodal part is formed by the slow Gc molecule populations reacting with (presumably) the same Gc-antibodies

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*FROM THE STATE INSTITUTE FOR BLOOD GROUP SEROLOGY, STATENS RÄTTSKEMISKA LABORATORIUM STOCKHOLM 60 (HEAD BIRGER BROMAN, M.D.) AND THE DEPARTMENT OF OBSTETRICS AND GYNECOLOGY KAROLINSKA SJUKHUSET STOCKHOLM 60 (HEAD ULF BORELL M.D.)*

# IMMUNO ELECTROPHORETIC STUDIES OF SERUM PROTEINS WITH PARTICULAR REFERENCE TO THE GC SYSTEM IN EXCHANGE-TRANSFUSED NEWBORNS

*A Preliminary Report*

*By*

JAN HIRSCHFELD and BO A NILSSON

Received 15 iv 62

Recent studies on human serum proteins by means of starch gel electrophoresis (12–14) and immunoelectrophoresis (1–9) have revealed the existence of proteins with varying electrophoretic mobilities in sera from different individuals Up to now, three independent protein systems the haptoglobins (Hp), the transferrins (Tf) and the group-specific components (Gc) have been found to occur in electrophoretically distinct and genetically determined types in individual sera (2, 12, 13)

These serum protein differences can be visualized by means of immunoelectrophoresis since precipitates with varying electrophoretic positions and shapes appear when sera belonging to different Hp-, Tf or Gc types are tested This fact allows sera to be divided into several main groups (2 9)

Sera which have a fast migrating group specific component (Gc 1-1) give an arc shaped precipitate which is situated more anodal than that of sera with the slow component (Gc 2 2) In addition certain human sera belong to a third main group (Gc 2 1) which in immunoelectrophoresis gives a two peaked or extended precipitate (2) This shape of the Gc 2 1 precipitate can be explained by the simultaneous presence of the 'fast' as well as the 'slow' Gc protein in sera of this type Therefore a similar precipitate can be obtained by subjecting a mixture of Gc 1 1 and Gc 2 2 sera to immunoelectrophoresis (2, 6, 7) The anodal part of this extended precipitate is thus formed by the react[illegible]

[illegible] (presumably) the same Gc antibodies

Thus, due to a "reaction of immunological identity" between these two immunologically identical but electrophoretically different molecule populations, two confluent precipitates are formed

In the present paper, the effect of blood transfusions on the Gc-pattern of the recipient will be *discussed*

## MATERIALS AND METHODS

Blood samples were drawn from 23 newborn infants of Rh immunized mothers at the beginning and end of exchange transfusions, and *in some instances at varying* intervals after the transfusion(s) The serum was decanted after incubation at +4° C overnight and kept at —20° C until investigated Blood from the donors used in the transfusions was treated in a similar manner

Immunoelectrophoresis was performed on object slides (11) according to a modified procedure described elsewhere (3) The patterns were developed with absorbed anti human immune sera prepared in rabbits (4 5) After electrophoresis the absorbing reactant and 1 hour later, the immune serum were added to the antibody basin and the slides incubated at 37° C for about 20 hours On the following day notes were taken of the results upon which the slides were photographed in oblique light

## RESULTS

The analysis of the samples showed that in some children the shape and electrophoretic position of the Gc-precipitates after exchange transfusion were different from findings before the transfusion

A representative case is shown in Fig 1 where the immuno electrophoretic patterns of several samples taken from the same child at varying intervals are presented (Table 1) The child's Gc type before transfusion is Gc 2 2 (Fig 1 A) Immediately after the first transfusion, his blood sample was classified as Gc 2–1 (Fig 1 B) In a new sample taken 16 hours later, the Gc-type was again shown to be Gc 2–2 (Fig 1 C) After the second exchange transfusion, the child's blood gave an asymmetrical precipitate when tested in immunoelectrophoresis (Fig 1 D) This type of precipitate is not found in non-transfused individuals

The various Gc patterns of the infant's serum are also shown in Table 1

Similar results were obtained for the other exchange- transfused infants Thus, children belonging to Gc 1–1, 2–1 or 2–2 after transfusion always gave Gc-precipitates with changed shapes if the donor belonged to a Gc-type different from the recipient, whereas if donor and recipient were of the same Gc-types, no change in shape of the Gc-precipitate was observed The 5 different shapes of the Gc-precipitate seen in connection with exchange transfusions are given in Table 2

In all instances in which the shape of the Gc-precipitate changed, these changed patterns were found to disappear within 20 hours after transfusion regardless of Gc-types of donor and recipient

In Table 3, the different Gc-types of the recipient (before and immediately after transfusion) and the donors found in the present material are given

TABLE 1

*Relation of Immuno Electrophoretic Patterns of Different Blood Samples from the Same Infant (Fig 1) with Certain Pertinent Anamnestic Data*

| Sample No | Fig | Time for transfusions and withdrawal of blood (hours) | Gc pattern of foetal blood | Gc-type of foetal blood | Gc type of transfused blood | Amount of transfused blood |
|---|---|---|---|---|---|---|
| 1 | 1 A | At birth (0) | slow normally arc shaped | 2 2 | – | – |
| | | Transfusion I (3 4) | – | – | Gc 1-1<br>Gc 2 1 | 520 ml s |
| 2 | 1 B | After transfusion I (4) | two peaked | 2 1 | – | – |
| 3 | 1 C | Before transfusion II (20) | slow normally arc shaped | 2 2 | – | – |
| | | Transfusion II (20 21) | – | – | Gc 2 2<br>Gc 1-1 | – |
| 4 | 1 D | After transfusion II (21) | asymmetrical | 1-1 + 2 2 | – | 540 ml s |

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In Table 3, the different Gc-types of the recipient (before and immediately after transfusion) and the donors found in the present material are given

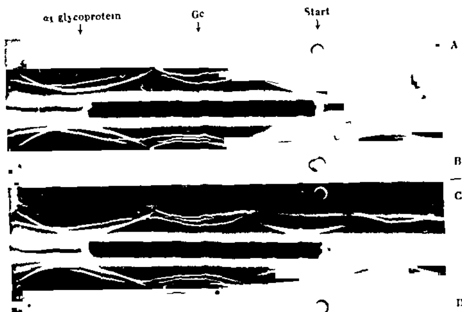



*Fig 1*

Immuno electrophoretic patterns of serum from a newborn taken at birth (A) after 1st exchange transfusion (B) before 2nd exchange transfusion (C) and after 2nd exchange transfusion (D) The precipitate closest to the antibody trough under arrow "Gc" is the Gc precipitate Note also absence of one strong precipitate (haptoglobin) in this area in A (umbilical cord serum) as compared to B C and D

and Gc 1 1 sera in equal amounts (Fig 1 B) The practical importance of these findings is *inter alia* that the disappearance of certain passively transferred blood proteins evidently can be followed by means of immunoelectrophoresis This is shown in Fig 1 C where the fast molecule populations are no longer demonstrable implicating their disappearance under the sensitivity limit of the technique within 16 hours after transfusion

This rapid disappearance of foreign proteins has also been observed for the other cases (Table 3) and for the haptoglobins (10)

In addition to these more qualitative aspects of transfused serum proteins a semiquantitative measure of the *ratio between the individuals* own protein and the transfused protein is obtained by immuno electrophoresis This is due to the fact that the position of a particular precipitate between the diffusion centers for antigens and antibodies varies with the antigen antibody ratio Thus when sera belonging to Gc 1 1 and Gc 2 2 are mixed in different relative proportions subsequent immuno-electrophoretic analysis of the mixtures will reveal Gc-precipitates with varying shapes (6 7) This effect is also *obtained* by mixing sera belonging to Gc 1 1 with sera of type Gc 2 1 (6 7) If *e g* sera belonging to Gc 1 1 and Gc 2 2 are mixed in the ratio of 1 1, a

TABLE 2
*Diagrammatic Drawings of the Different Gc Precipitates Found in the Present Material*

| Precipitate shape | Anode to the left |
|---|---|
| 1 | |
| 2 | |
| 3 | |
| 4 | |
| 5 | |

TABLE 3
*Different Combinations of Gc Patterns of Recipients (before and immediately after Transfusion) and Donors Found in the Present Material (Figures in Brackets Refer to the 5 Different Shapes of the Gc-precipitate as given in Table 2)*

| Gc type of recipient | | Gc type of donor |
|---|---|---|
| before | after | |
| 1 1 (4) | 1 1 (4) | 1 1 (4) |
| 1 1 (4) | asymm (2) | 2 1 (3) |
| 2 1 (3) | asymm (2) | 1 1 (4) |
| 2 1 (3) | 2 1 (3) | 2 1 (3) |
| 2 1 (3) | asymm (1) | 2 2 (5) |
| 2 2 (5) | 2 1 (3) | 1 1 (4) |
| 2 2 (5) | asymm (1) | 2 1 (3) |

## DISCUSSION

The general results given in Table 3 show that immuno-electrophoretic analysis of serum from transfused recipients allows the recognition of foreign Gc protein if the blood is taken immediately after transfusion This feature seems to apply also to transfused, adult individuals In the example given in Fig 1 and Table 1, the Gc 1 1 molecule populations in the donors used in exchange transfusion I are demonstrable after transfusion in the child's serum Together with the infant's own Gc molecule populations, they give a pattern indistinguishable from the precipitate of Gc 2–1 individuals or from an in vitro mixture of Gc 2 2

transferred foreign serum proteins in the host and allow a further insight into some of the many complex aspects of blood transfusion

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symmetrically extended or two-peaked precipitate is obtained If, however, the mixture is carried out in the ratio 1 3, the Gc-precipitate is asymmetrical with a shape similar to that of Fig 1 D (7) The explanation of this is that the concentration of the fast Gc molecule population is only 1/3 of the concentration of the slow Gc-protein in such mixture Consequently, the Gc-precipitate caused by the reaction of the fast Gc molecules with the antibodies in the immune serum will be formed closer to the antigen diffusion center than that formed by the slow Gc molecules which, because of a higher concentration of antigen, will appear closer to the antibody basin Therefore, the shape of a precipitate in immunoelectrophoresis will reflect the quantitative distribution of electrophoretically different although immunologically identical molecule populations in the gel if it is assumed that these different molecule populations are identical in other respects concerning, *e g*, their diffusibility in gel, their qualitative degree of reactivity with the antibodies, etc It has been shown by artificial mixture experiments that these assumptions are valid for the fast and slow molecule populations of the group specific components when tested against hetero-immune sera prepared in rabbits, horses, and monkeys (6, 7)

The asymmetrical shape of the Gc-precipitate in Fig 1 D might, therefore, be explained by assuming that a smaller amount of fast Gc molecule populations is present in this sample as compared to its content of slow Gc protein The reason for this is the fact that the donors in exchange transfusion II were of the types Gc 2 2 and Gc 2–1 Consequently, the amount of the fast Gc protein (1–1) which has been transfused is smaller than the amounts of the transfused and the individual's own Gc 2–2 protein

In conclusion, the demonstration of serum protein polymorphisms is not only of value as regards their use in genetic studies but also represents a "physiological tagging' by means of which passively transferred proteins can be identified and studied with regard to quantitative and qualitative properties in the host In the present paper, the validity of this postulate has been demonstrated and tentative, practical applications of the Gc-system in connection with transfusions suggested


SUMMARY

Sera from passively immunized newborns were studied by means of immunoelectrophoresis The samples were taken from the same individual before and after exchange transfusions In some instances, pronounced changes in shape and electrophoretic position of a polymorphous protein precipitate (Gc) were found Some of these changed immuno-electrophoretic patterns are shown and the underlying mechanism discussed It is concluded that the occurrence of genetically determined, different serum proteins in different individuals might be of value in studies of quantitative and qualitative aspects of passively

transferred "foreign" serum proteins in the host and allow a further insight into some of the many complex aspects of blood transfusion

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vaccinia vaccination using multiple pressure technique Blood samples were taken in the first week i e before the vaccinations and in the fourth and seventh weeks

the same person were always tested simultaneously

The positive results recorded are shown in the table A transient appearance of the RF as measured by the Waaler Rose and/or latex one tube test was observable in eight instances There were two additional cases with titer increase but no reduction In three instances positive results remained stationary through the observation period No cases with positive results in only the first sample were recorded All the recruits with positive results were in good health during the observation period Thus it seems reasonable to correlate the appearance of the RF with vaccinations Whether the results reflect a true appearance of the RF in the circulation or only a titer increase above the sensitivity threshold of the methods cannot be said with certainty It is suggested that among apparently healthy persons there are a few with varying degrees of a tendency to form RF in connection with antigen stimulation

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## BRIEF REPORT

### A MOUSE PATHOGENIC STRAIN OF PARA INFLUENZA VIRUS TYPE 3 ISOLATED FROM CATTLE

by *C Rindom Schiøtt* and *C Hyldgaard Jensen*

Several viruses causing respiratory infections in human beings have been isolated from cattle *Klein et al* (1959 1960) isolated a bovine adenovirus related to

From a cow with mucosal disease we have recently isolated a virus designated

Material from rectal and nasal swabs from the cow was inoculated in kidney tissue cultures and after 4 days a cytopathogenic effect (CPE) was observed in the cultures inoculated with material from the nose Titration of virus infected

$10^{-3}$ $TCD_{50}$ per 0 1 ml

laques with degenerated cells and later

msa stained preparations a number of

s found to pass Mill pore filters with a pore size of 450 mμ but not filters with 100 mμ a p d The virus gave an agglutination of red cells from cow guinea pig and human type O Further the infectivity of virus KO 23 was destroyed by exposure to 20 per cent anesthetic ether for 24 hours at +4° C

Received 4 xii 62 *from the Department of Neurology Aarhus Kommunehospital and the Institute of General Pathology University of Aarhus Denmark*

# BRIEF REPORT

## TRANSIENT APPEARANCE OF THE RHEUMATOID FACTOR IN CONNECTION WITH PROPHYLACTIC VACCINATIONS

By K. Aho[1,2], A. Konttinen, M. Rajasalmi and O. Wager[1]

It has been possible to produce a rheumatoid factor like substance in rabbits by antigen stimulation. In addition to chronic liver diseases, false positive results in rheumatoid serological tests have been encountered in various infections. Recently a high percentage of positive reactions was recorded by *Williams & Kunkel (3)* in long lasting cases of subacute bacterial endocarditis.

These observations suggest that the appearance of the rheumatoid factor (RF) is connected with *in vivo* antigen antibody interactions. However, for explanation of the relatively high specificity of the RF there remains the question of why it should be assumed that the antigen antibody interactions in rheumatoid arthritis would differ essentially from those, *e.g.*, in rheumatoid spondylitis or in lues (in long lasting cases of subacute bacterial endocarditis the antigen stimulation apparently is very strong).

TABLE. *Recorded Positive Results*

| Name | Rheumatoid Factor (Waaler-Rose Latex) | | | |
|---|---|---|---|---|
| | 1st Sample | 2nd Sample | 3rd Sample | 4th Sample |
| Ja | 0/ | 0/+ ? | 0/ | |
| Ka | 0/ | 500/ | 128/+ | 32/ |
| Ko[1] | 0/ | 64/ | 0/ | |
| Mc | 0/ | 0/+ | 0/ | |
| Ri | 0/ | 128/+ | 0 + | 0/ |
| Sa | 0/ | 0/+ ? | 0/ | |
| Uk | 0/– | 0/+ ? | 0/ | |
| Vi | 0/ | 0/+ | 0/ | |
| An | 0/ | 0 | 0/+ ? | |
| Im | 32/ | 64/+ | 64 + | 64 + |
| Le[2] | 0/+ ? | 0/+ ? | 0/+ | |
| Aa | 128/+ | 128/+ | 128/+ | |
| Wa | 250/+ | 250/+ | 500/+ | |

1 Occasional minor subjective complaints in proximal interphalangeal joints
2 Mother suffering from rheumatoid arthritis

In order to evaluate the possible role of the host response, we have followed the serological events in a series of 245 healthy military recruits. During the second and third weeks of their service they were subjected to the following vaccination program: Combined tetanus and Salmonella vaccine (5 Lf of tetanus toxoid and $10^{8.4}$ S. typhi, $10^{8.3}$ S. paratyphi B and $10^{8.1}$ S. typhimurium), mumps vaccine (128 hemagglutinating units of formaline inactivated Enders strain), diphtheria vaccine (1 Lf of toxoid), polio vaccine ($10^{8}$ *type I*, $10^{5}$ *type II* and $10$ *type III*) and

Received 29. xi. 62 *from the Municipal Bacteriological Laboratory, Aurora Hospital, Helsinki, Finland*.
1 Supported by research grant A 3026 from the *National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service*.
2 Supported by a grant from the *Sigrid Jusélius Foundation*.

The prevalence of virus KO 23 antibodies in the sera of cattle was studied from October 1961 to June 1962 Acute and convalescent sera (80 pairs) were obtained from cattle in herds with present or previous experiences of mucosal disease 95 per cent of the acute and all of the convalescent sera were able to neutralize virus KO 23 *The significance of the increasing amount of antibodies against virus KO 23 in relation to disease will be discussed elsewhere*

Sera from healthy human beings also reveal antibodies in a large proportion of cases One group consisted of 49 sera from healthy donors 18 56 years old and the other sera (48) were taken from children 6 month to 15 years old Only 2 of the adults had no antibodies in their serum (diluted 1/8) as compared to 10 of the children About half of the sera from adults and 25 per cent of the childrens sera were able to neutralize 300 $TCD_{50}$ of virus KO 23 when the serum was diluted 1/128 or more (*Rindom Schiott*)

The CPE the filtration experiments the hemagglutination capacity and the sensitivity to ether render it likely that virus KO 23 is related to the para influenza group of viruses Virus KO 23 has infact later been identified as the bovine type of myxovirus para influenza type 3 by the hemagglutination inhibition test and we are indebted to Robert M Chanock MD NIH Washington DC who kindly made the identification

In one respect strain KO 23 differs from other PI 3 strains In our first report we found that strain KO 23 was pathogenic for suckling mice after intracerebral inoculation Injection of 0 01 ml of undiluted tissue culture fluid containing $10^{5\ 8}$ $TCD_{50}$ of virus resulted in infection 6 7 days later The mice were apathic and looked thinner and smaller than the animals of the control group The gait was considerably more reeling and shaky than normal Paralysis did not occur Attempts to pass the virus by intracerebral inoculation of brain material from sick animals were unsuccessful

Later we have succeeded in passing the virus by the intracerebral route for more than 10 passages using a 10 per cent brain suspension as passing material When this material is titrated in tissue culture it gives from $10^{2\ 8}$ to $10^{3\ 8}$ $TCD_{50}$ per 0 1 ml

After each passage the virus has been identified by neutralization with an immune serum prepared by inoculation of guinea pigs with virus KO 23 infected tissue culture fluid Thus the possibility of a contamination with an unrelated mouse pathogenic virus can be excluded

The experimental infection with this mouse adapted PI strain will be further investigated

*Summary* The isolation of a virus from a cow with mucosal disease is described The virus has been identified as a bovine type of myxovirus para influenza type 3 The virus is pathogenic for suckling mice after intracerebral inoculation

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